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Cover: Fluorescent microscopy image of osteoblasts adhering on amine reactive OptiChem®.

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Para mi familia

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Chapter 1

General Introduction

Biomaterial-associated infections

The use of synthetic materials in prosthetics, artificial organs and biomedical devices in general has become a widespread practice in modern medicine. However, biomaterial associated infections (BAI) and deficient tissue integration are well-known problems that often limit their application and represent a threat to the patient's health and life as well as for the implant's longevity and functionality. The incidence of BAI varies per implanted device and site. For example, 0.3 to 5 % of all orthopedic implants[1,2] and 0.1 to 70 % of nonvalvular cardiovascular devices,[3] are subject to BAI. BAI is often caused by non-pathogenic bacteria, such as commensals from the skin (e.g. *Staphylococcus epidermidis* and *Staphylococcus aureus*), which can contaminate an implant during insertion, but once adhering to a biomaterial's surface become virulent.[4,5] Despite improved sterile and surgical techniques, peri-operative contamination remains the main route of BAI. BAI caused by post-operative contamination and contamination through compromised local tissues or blood stream are less frequent[1,4,5]

The first step in the development of BAI is bacterial adhesion. Bacteria adhering to an implanted device grow and colonize the surface while producing extracellular polymeric substances (EPS) to form biofilm. The biofilm mode of growth constitutes a protection for bacteria to survive in hostile milieus with respect to their planktonic forms, and allows them to evade the host immune system.[6,7] Biofilms represent a challenge for physicians as biofilms are more resistant to antimicrobial therapy than planktonically growing organisms. Treatment generally involves the removal of the implant from the infected tissue followed by systemic antimicrobial therapies to clear the infection from surrounding tissues at substantial healthcare cost, patient discomfort, and high morbidity and mortality rates. In many cases, the prospects of a revision surgery are lower than those of any primary implant because the surrounding tissue may remain compromised by bacterial presence.[8]

The susceptibility of biomaterials for BAI depends on the interaction between biomaterial, microorganisms and host cells. The biomaterial surface dictates the fate of the implanted device, i.e., if the biomaterial surface promotes endogenous host cell spreading and proliferation, it is likely that the implanted device will successfully

integrate within the host tissue, while it makes the surface less prone to bacterial colonization and biofilm formation.[4]

During implantation of an indwelling device, tissue trauma and injury modulate a series of events which involve host cells and the immune system. Neutrophils and macrophages are the predominant infiltrating cells that arrive at the implant site within hours after implantation.[9,10] Contrary to neutrophils, macrophages proliferate notably and can remain at the implant surface for several weeks. Macrophages are responsible for inflammatory reactions, repair and eventually foreign body responses, but are also important components in the defense against microbial infection,[9] including BAI. When tissue is infected, macrophages detect pathogens and adhere to their surface and subsequently engulf bacteria and trigger cellular functions to destroy them and recruit other cells from the adaptive immune system.[11,12] However, the presence of biomaterials can limit macrophage migration and phagocytic activity, enabling bacteria to survive.[13]

Strategies to prevent BAI

Adhesion of bacteria to biomaterial surfaces is the first step and an essential factor in the development of biofilms. Controlling this process can contribute to reduce the risk of BAI. For this purpose, biomaterials can be modified with surface coatings that change their physico-chemical properties and discourage non-specific interactions between bacteria and the surface of the implant. The most extensively studied strategies include the use of low surface free energy coatings also known as hydrophobic coatings,[14,15] positively charged coatings,[16] quaternary ammonium compounds,[17] and polymer brushes.[18-21] Polymer brushes are being promoted as one of the most promising methods to reduce biomaterial-centered infections.[22,23] These coatings have a high capacity to reduce protein adsorption and bacterial and tissue cell adhesion. For instance, polymer brushes made of polyethylene glycol have been shown to reduce bacterial adhesion several orders of magnitude more than any other anti-adhesive coatings.[22,23]

Polymer brush coatings are made of highly mobile polymer chains which are tethered by one end to a surface or interface at a high density (i.e., very small distances between neighboring anchored chains ends). As a result from the high density a steric repulsion originates from the osmotic pressure inside the brushes that causes the chains to stretch away from the surface to the intervening medium forming a brush-like configuration.[18,20] This steric barrier makes adsorption of proteins, microorganisms or cells approaching the surface thermodynamically difficult and therefore adhesion is weak.[18,20,24] A schematic representation of a bacterium approaching a polymer brush coating is shown in Figure1.

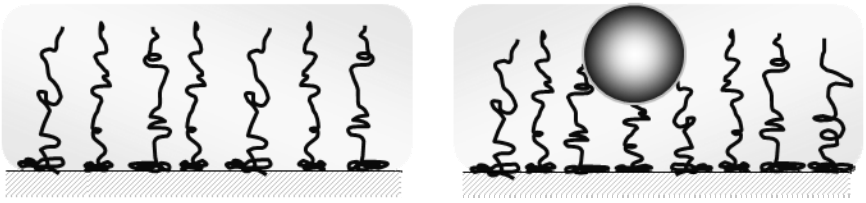

Figure 1. Schematics of a polymer brush preventing bacterial adhesion.

An additional feature of polymer brushes is that they are often highly hydrated. Together with the weak interaction forces exerted by polymer brushes, this makes them "biologically invisible" ("stealth coatings)".

OptiChem® as a potential coating to prevent BAI

OptiChem® is a commercially developed poly(ethylene) glycol (PEG) based brush-like polymer coating designed to inhibit non-specific biomolecular adsorption, protein and cell binding. By design, the polymer surface chemistry can be chemically modified to allow specific covalent immobilization of molecules within the same low non-specific binding coating matrix.[25-27] This is specifically desired for biomaterial applications since it would be optimal for performance if bacterial adhesion is inhibited while the same coating promotes and supports cellular adhesion.

Polymer coating formulation

The coating chemistry comprises three core components: an active component, a matrix-forming component and an intermolecular cross-linking component.[25-27] The active base component is a hetero-bifunctional PEG molecule (molecular weight = 3400) terminated with a succinimidyl ester (NHS) which serves as a functional group in the final coating, and an alkoxysilane terminus that functions as a reactive crosslinking group, providing covalent attachment within the coating matrix and to certain substrates. The matrix-forming ethylene glycol oligomer component is a non-ionic surfactant containing ethylene oxide repeating units (polyoxyethylene sorbitan tetraoleate). The intermolecular cross-linking component is an azidosilane molecule. All three components are mixed in an organic solution. Upon thermal or photo- activation after coating, the azido group inserts into aliphatic or aromatic bonds within the coating matrix or on organic substrates; the silane end crosslinks with other silanes in the matrix and provides covalent linkage to surface oxides on certain substrates. These three primary matrix components crosslink together within a volatile carrier solvent and attach covalently upon curing to surfaces. A representation of the coating architecture and three coating components is presented in Figure 2. The result of this process is a robust and optically transparent thin polymer-based film, with numerous functional coupling chemistry and bio-immobilization capabilities.

OptiChem® coatings provide a PEG-tethered NHS reactivity after cure, to allow specific attachment of certain nucleophilic molecules (e.g. reactive amines, see Fig. 2), which makes them suitable for cellular adhesion applications using immobilized cell adhesion peptides and selected cell matrix proteins.[25-27] For non-adhesive purposes, the NHS groups can be deactivated throughreaction of these reactive end groups by small molecule nucleophiles by submerging the coated slides in methoxyethylamine and borate buffer. The primary amine terminus reacts with the NHS groups in the coating to create amide-linked ethyl methoxy groups terminating the crosslinked PEG chains.

Distinct from other PEG-based coatings, OptiChem® can be applied on a variety of solid substrata in a single step with conventional industrial techniques such as spin coating, dip coating and spraying.

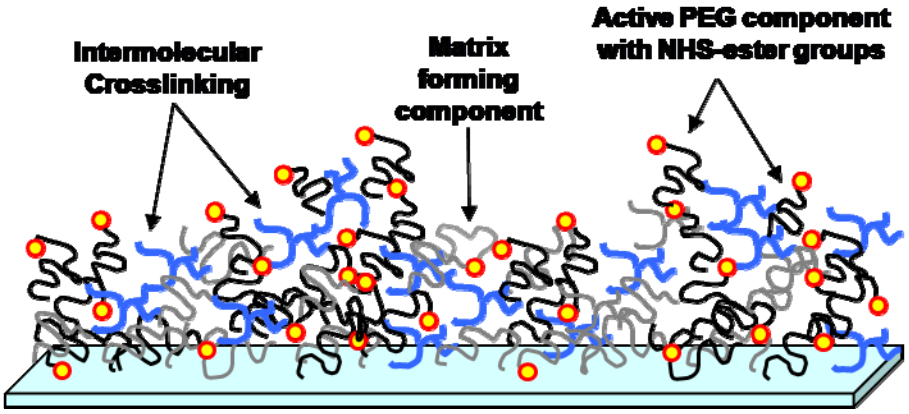

Figure 2. OptiChem® coating architecture and surface chemistry components.

Aim of this thesis

Due to the coating properties, the general aim of this thesis is to investigate the extent up to which Optichem®-based coatings can contribute to the prevention of BAI.

References

1. Del Pozo J.L, Patel R. Infection associated with prosthetic joints. *N Engl J Med* 2009; 361:787-794.
2. Campoccia D, Montanaro L, Arciola CR. The significance of infection related to orthopedic devices and issues of antibiotic resistance. *Biomaterials* 2006; 27:2331-2339.
3. Baddour LM, Bettmann MA, Bolger AF, Epstein AE, Ferrieri P, Gerber MA, Gewitz MH, Jacobs AK, Levison ME, Newburger JW, Pallasch TJ, Wilson WR, Baltimore RS, Falace DA, Shulman ST, Tani LY, Taubert KA. Nonvalvular cardiovascular device-related infections. *Clin Infect Dis* 2004; 38:1128-1130.
4. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* 1987; 237:1588-1595.
5. Schierholz JM, Beuth J. Implant infections: a haven for opportunistic bacteria. *J Hosp Infect*. 2001; 49:87-93.
6. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2004; 2: 95-108.

7. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002; 15:167-193.

8. Broekhuizen CA, de Boer L, Schipper K, Jones CD, Quadir S, Vandenbroucke-Grauls CM, Zaat SA. *Staphylococcus epidermidis* is cleared from biomaterial implants but persists in peri-implant tissue in mice despite rifampicin/vancomycin treatment. *J Biomed Mater Res A* 2008; 85:498-505.

9. Anderson JM. Biomaterials Science. An Introduction to Materials in Medicine. Elsevier San Diego, CA, pp 296-304 Inflammation, wound healing, and the foreign-body response. In: Ratner BD. Hoffman AS, Schoen FJ, Lemons JE, editors. Biomaterials Science. An Introduction to Materials in Medicine. San Diego, CA: Elsevier; 2004. pp 296-304.

10. Lindblad M, Lestelius M, Johansson A, Tengvall P, Thomasen P. Cell and soft tissue interactions with methyl- and hydroxylterminated alkane thiols on gold surfaces. *Biomaterials* 1997; 18:1059-1068.

11. Berton G, Lowell CA. Integrin signalling in neutrophils and macrophages. *Cell Signal* 1999; 11: 621-635.

12. Colditz IG, Watson DL. The effect of cytokines and chemotactic agonists on the migration of T lymphocytes into skin. *Immunology* 1992; 76:272-278.

13. Boelens JJ, Dankert J, Murk JL, Weening JJ, Van der Poll T, Dingemans KP, Koole L, Laman JD, Zaat SAJ. Biomaterial-associated persistence of *Staphylococcus epidermidis* in pericatheter macrophages. *J Infect Dis* 2000; 181:1337-1349.

14. Everaert EPJM, Van der Mei HC, Busscher HJ. Adhesion of yeasts and bacteria to fluoro-alkylsiloxane layers chemisorbed on silicone rubber. *Coll Surfaces B: Biointerfaces* 1998;10: 179-190.

15. Tsibouklis J, Stone M, Thorpe AA, Graham P, Peters V, Heerlien R, Smith JR, Green KL, Nevell TG. Preventing bacterial adhesion onto surfaces: the low-surface-energy approach. *Biomaterials* 1999; 20:1229-1235.

16. Gottenbos B, Grijpma DW, Van der Mei HC, Feijen J, Busscher HJ. Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria. *J Antimicrob Chemother* 2001; 48:7-13.

17. Tiller JC, Liao CJ, Lewis K, Klibanov AM. Designing surfaces that kill bacteria on contact. *Proc Natl Acad Sci USA* 2001; 98:5981-5985.

18. Norde W, Gage D. Interaction of bovine serum albumin and human blood plasma with PEO-tethered surfaces: influence of PEO chain length, grafting density, and temperature. *Langmuir* 2004; 20:4162-4167.

19. Desai NP, Hossainy SF, Hubbell JA. Surface-immobilized polyethylene oxide for bacterial repellence. *Biomaterials* 1992; 13:417-420.

20. Currie EP, Norde W, Cohen SMA. Tethered polymer chains: surface chemistry and their impact on colloidal and surface properties. *Adv Coll Interf Sci* 2003; 100:205-265.

21. Kingshott P, Wei J, Bagge-Ravn D, Gadegaard N, Gram L. Covalent attachment of poly(ethylene glycol) to surfaces, critical for reducing bacterial adhesion. *Langmuir* 2003; 19:6912-6921.

22. Roosjen A, Norde W, Van der Mei HC, Busscher HJ. The use of positively charged or low surface free energy coatings versus polymer brushes in controlling biofilm formation. *Prog Coll Polym Sci* 2006; 132:138-144.

23. Nejadnik MR, Van der Mei HC, Norde W, Busscher HJ. Bacterial adhesion and growth on a polymer brush-coating. *Biomaterials* 2008; 29:4117-4121.

24. Leckband D, Sheth S, Halperin A. Grafted poly(ethylene oxide) brushes as nonfouling surface coatings. *J Biomater Sci Polym Ed* 1999; 10:1125-1147.

25. Mao G, Metzger S, Lochhead MJ (2005) US patent 6,844,028, 2005.

26. Harbers GM, Emoto K, Greef C, Metzger SW, Woodward HN, Mascali JJ. Functionalized poly(ethylene glycol)-based bioassay surface chemistry that facilitates bio-immobilization and inhibits nonspecific protein, bacterial, and mammalian cell adhesion. *Chem Mater* 2007; 19:4405-4414.

27. Takahashi H, Emoto K, Dubey M, Castner DG, Grainger DW. Imaging surface immobilization chemistry: correlation with cell patterning on non-adhesive hydrogel thin films. *Adv Funct Mater* 2008; 18:2079-2088.

Chapter 2

The Risk of Biomaterial-Associated Infection after Revision Surgery due to an Experimental Primary Implant Infection

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Anton F. Engelsman, Isabel C. Saldarriaga Fernández, M. Reza Nedjanik, Gooitzen M. van Dam, Kevin P. Francis, Rutger J. Ploeg, Henk J. Busscher, Henny C. van der Mei.

Abstract

The fate of secondary implants was determined by bio-optical imaging and plate counting, after antibiotic treatment of biomaterials-associated-infection (BAI) and surgical removal of an experimentally infected, primary implant. All primary implants and tissue samples from control mice showed bioluminescence and were culture-positive. In an antibiotic treated group, no bioluminescence was detected and only 20% of all primary implants and no tissue samples were culture-positive. After revision surgery, bioluminescence was detected in all control mice. All of the implants and 80% of all tissue samples were culture-positive. In contrast in the antibiotic treated group, 17% of all secondary implants and 33% of all tissue samples were culture-positive, despite antibiotic treatment. The study illustrates that the infection risk of biomaterial implants is higher in revision surgery due to BAI of a primary implant than in primary surgery, emphasizing the need for full clearance of the infection, also from surrounding tissues prior to implantation of a secondary implant.

Introduction

Infections associated with implanted biomaterials are a frequently occurring problem in modern surgery. Antibiotic treatment is considered a cornerstone in the treatment of biomaterials-associated infection (BAI), but is often unsuccessful and may be followed by surgical removal of the primary and insertion of a secondary implant. Yet, the outcome of revision surgery after BAI is quite uncertain, which increases the length of hospital-stays and associated costs.[1] BAI is typically caused by commensal bacteria, e.g. *Staphylococcus aureus*, which adhere to the biomaterial surface and produce extracellular polymeric substances to form a biofilm on the implant surface.[2] The biofilm mode of growth provides a reduced bacterial susceptibility to antimicrobial agents.[3] BAI is usually treated with vancomycin, often in combination with rifampicin. Vancomycin is known to effectively penetrate biofilms and substantially reduce the number of viable bacteria.[4] Yet, vancomycin treatment has a relatively high failure rate, which can be explained in part by low metabolic activity of bacteria in a biofilm.[5] Broekhuizen *et al*. and Boelens *et al*. showed that bacteria can also be located inside macrophages surrounding a biomaterials implant, where they remain protected against antibiotic treatment.[6-8] Thus, both the biofilm mode of growth on the surface of a biomaterial implant as well as the bacterial localization in peri-implant tissues offer protection to the bacteria involved in BAI against routine antibiotic treatment, which may compromise the outcome of revision surgery.

When the aspects described above are taken into account, primary implants can be expected to encounter a different risk of infection from BAI than secondary implants after revision surgery. Primary implants are at risk of becoming infected during operation and sometimes hospitalization or by hematogenous spreading of bacteria from infections elsewhere in the body.[9-11] Bacteria infecting a secondary implant may arise, however, from peri-implant tissue and usually have been exposed for longer periods of time to antibiotics, possibly creating resistance or altering their adhesiveness for an implant surface. [12]

Silicone rubber is a hydrophobic material, which is typically used in catheter systems and flexible implants such as vocal, breast and penile prostheses. Clinically, it is known

that the risks of infection of a secondary implant after primary BAI are much higher than those of a primary implant, but rigorous numbers of the infection risk in revision surgery after BAI are not available. One of the few reports published, mentions that while 1–3% of primary penile prostheses and urinary sphincters become infected, infection percentages after BAI increase to 9% in revision surgery. [13] Infections during revision surgery are notorious for their progressive resistance to the antibiotic regimen due to changes in bacterial resistance patterns.[14] Research so far has focused on the prevention of infection of primary implants, despite the fact that infections of secondary implants after BAI of a primary implant occur more frequently.

In vivo imaging is currently rapidly emerging as a technique to longitudinally monitor BAI in living animals.[15-18] The main advantage of *in vivo* imaging is that it allows the spatiotemporal monitoring of bacterial persistence without sacrificing the animal. *In vivo* imaging has been used in a number of *in vivo* infection studies to evaluate efficacies of antibiotic regimens against BAI.[16,17,19-21] The aim of this study is to determine the fate of a secondary silicone rubber implant, when inserted in an infected pocket, after routine treatment of primary BAI with antibiotics and surgical removal of the infected primary implant. Experiments were carried out in immuno-competent mice and BAI was monitored *in vivo* using a bioluminescently reporting *S. aureus* strain. In addition, bacterial presence in peri-implant tissues and on the silicone rubber implant was evaluated separately *ex vivo* by plate-counting.

Materials and methods

Biofilm formation by bioluminescent S. aureus *Xen29*

S. aureus ATCC12600 was made bioluminescent by stably integrating a modified *lux* operon into its chromosome, as described previously[16,17] and named Xen29. The strain was obtained commercially from Xenogen Corporation (now part of Caliper Life Sciences, Hopkinton, MA, USA). *S. aureus* Xen29 was cultured from cryopreservative beads (Protect Technical Surface Consultants Ltd., Lancashire, UK) onto a blood agar plate at 37°C in ambient air. One colony was used to inoculate 10 ml tryptone soy broth

(TSB, Oxoid, Basingstoke, UK) and grown overnight (16 h). To form a biofilm, a test tube with 10 ml TSB enriched with 4% NaCl was inoculated with 100 µl of the overnight culture (about 10^9 CFU ml^{-1} as separately determined by plate counting) in which a single sterile silicone rubber disc (diameter 8 mm; thickness 0.5 mm, Medin, Groningen, The Netherlands) was incubated for 72 h at 37°C on a rotary shaker (60 rpm) to grow a biofilm upon.

Implantation procedure of primary silicone rubber disc and initiation of BAI

Silicone rubber discs with biofilms were implanted in the left flank of 20 female Balb/c OlaHsd (Harlan Netherlands BV, Horst, The Netherlands) mice. Anesthesia was induced with 3.5% Isoflurane/O_2 (Zeneca, Zoetermeer, The Netherlands) gas mixture and maintained at 1.5% during the entire implantation procedure. In addition, buprenorfine (0.03 mg kg^{-1}) was administered subcutaneously 30 min in advance of the procedure as an analgesic. Prior to implantation of the contaminated silicone rubber discs, the left flank was shaved and cleaned with 70% ethanol. A 2 cm deep subcutaneous pocket was made through a 1 cm incision, in which one silicone rubber disc was placed. The incision was closed with a single 7-0 monofilament polypropylene (Surgipro™, US Surgical Corp., Norwalk, Connecticut, USA) suture. The discs were left *in situ* for 4 days, after which the pocket was opened under sterile conditions, using the same analgesia and anesthesia procedures as described for the initial implantation procedure. During this 4-day period, the infection was either treated with intraperitoneal antibiotics on a daily basis or with 0.9% NaCl. Based on an average bodyweight of the mice of 20 g, 0.5 ml of an antibiotic solution of 2 mg ml^{-1} vancomycin (vancomycin 500, Abbott bv, Hoofddorp, The Netherlands) + 1 mg ml^{-1} rifampicin (Rifadin, Aventis, Hoevelaken, The Netherlands) in 0.9% NaCl was injected intra-peritoneally.[8] Control mice received injections of 0.5 ml of 0.9% NaCl. These experiments were approved by the Animals Experiments Committee at the University Medical Center of Groningen.

Revision surgery and placement of secondary silicone rubber discs

At day 4, antibiotic and control treatments were ended and the primary silicone rubber discs were collected for *ex vivo* analyses. Six out of ten animals per group received a

sterile, secondary silicone rubber disc without a biofilm, while the wounds of the remaining animals were closed without placement of a new implant. At day 10, the secondary implants were removed along with a sample of tissue surrounding the implant site for further *ex vivo* analysis (see Figure 1 for an outline of the experiments). From the animals without a secondary disk a tissue sample from the primary implant site was taken after 10 days.

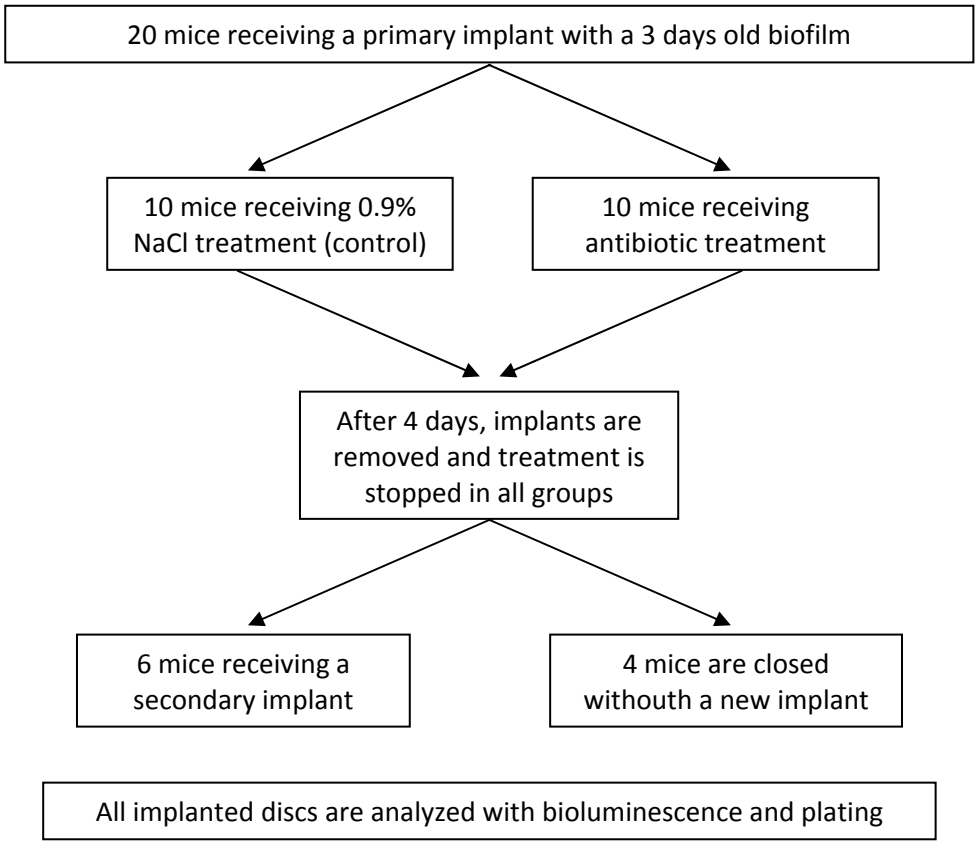


Figure 1. Overview of the experiments carried out.

Bioluminescence imaging

Bioluminescent imaging was used to evaluate the progression of BAI of the primary implant and to monitor the fate of the secondary implant. The bioluminescent signal was scanned in situ using a CCD camera (IVIS® Spectrum Imaging System, Caliper Life Sciences, Hopkinton, MA, USA). After acquiring a grey-scale photograph, a bioluminescent image was obtained using 15 cm field of view, binning of 4, 1/*f* stop and

open filters. The duration over which imaging was executed depended on the signal intensity which resulted in an average imaging duration of 2 min. The signal was considered as below threshold when no signal was obtained during a maximum imaging duration of 10 min. In case of a positive signal, regions of interests (ROIs) were defined by using a threshold of 600 photon counts over the total imaging duration, which is the minimal operating sensitivity of the IVIS. Bioluminescence was quantified by using radiance ($p/s/cm^2/sr$).

Ex vivo *quantification of bacteria on primary and secondary silicone rubber discs and in surrounding tissue*

Immediately after removal, the collected silicone rubber discs or tissue samples (approximate weight 2 g each) were transferred to the laboratory in Eppendorf tubes containing 1 ml reduced transport fluid (RTF: NaCl 0.9 g l^{-1} $(NH_4)_2SO_4$. 0.9 g l^{-1}, KH_2PO_4 0.45 g l^{-1}, Mg_2SO_4 0.19 g l^{-1}, K_2HPO_4 0.45 g l^{-1}, Na_2EDTA 0.37 g l^{-1}, L-cysteine HCl 0.2 g l^{-1}, pH 6.8). Staphylococci adhering to the discs were detached into suspension by intermittent sonication for three times 10 s at 30 W (Vibra Cell model 375; Sonics and Materials, Danbury, CT, USA). This procedure was found not to cause cell lysis or killing. Subsequently, this suspension was diluted and 100 µl was spread on blood agar plates and the numbers of colony forming units (cfu) were determined after incubation for 24 h at 37°C. Bacterial presence in tissues was determined after homogenization of the tissue in RTF by intermittent sonication for three times 10 s, subsequent serial dilution and culturing of 100 µl of the homogenate on blood agar plates. CFU's were enumerated as described above and normalized for the weight of the tissue sample.

MIC values of* S. aureus *Xen29 against vancomycin and rifampicin

In order to determine the minimal inhibitory concentrations (MIC) of bioluminescent *S. aureus* Xen29 against the two antibiotics used, staphylococci were exposed to rifampicin and vancomycin E-tests® (AB Biodisc, Solna, Sweden) according to the manufacturer's protocol. After 24 h growth at 37°C, MIC values were read from the E-test® strip. In addition, bioluminescent images of the agar plates were taken with an IVIS Spectrum along with a regular light photograph.

Statistics

Data were analyzed using the Statistical Package for the Social Sciences (SPSS 16.0 for Windows, Chicago, IL). The Mann-Whitney Rank test was used for comparison of the groups of the CFU numbers between the saline and antibiotic treated group. P-values < 0.05 were considered to indicate significant differences.

Results

Progression of primary implant infection

The presence of an infected primary silicone rubber disc yielded a clear bioluminescent signal in all mice treated with saline as a control (see a representative example in Figure 2A), but in the antibiotic treated group the bioluminescent signal was below the threshold value (Figure 2A). Removal of the primary implant (at day 4) induced an almost immediate and significant decrease of the bioluminescent signal in all mice treated with saline to below levels of detection (see Figure 2B for average data). *Ex vivo* quantification of bacterial presence using plate counting (Figure 2C) indicated bacterial presence on all silicone rubber discs in the saline group, while in the antibiotic-treated group only 20% of the removed primary implants appeared infected with *S. aureus* Xen29. The difference between the saline and antibiotic treated group is significant. After 10 days, tissue taken from the primary implant site was analyzed for the presence of bacteria by plate counting. All excised tissue samples in the saline-treated group were culture positive for *S. aureus* Xen29, while none of the tissue samples from the antibiotic-treated group yielded any bacteria which was not significant due to the small group size (see Figure 2C).

Infection of the secondary implant

After revision surgery at day 4, bioluminescence could be quantified *in vivo* in saline-treated mice with a secondary implant, but bioluminescence remained below threshold in the antibiotic-treated group with a secondary implant (see Figures 3A and B).

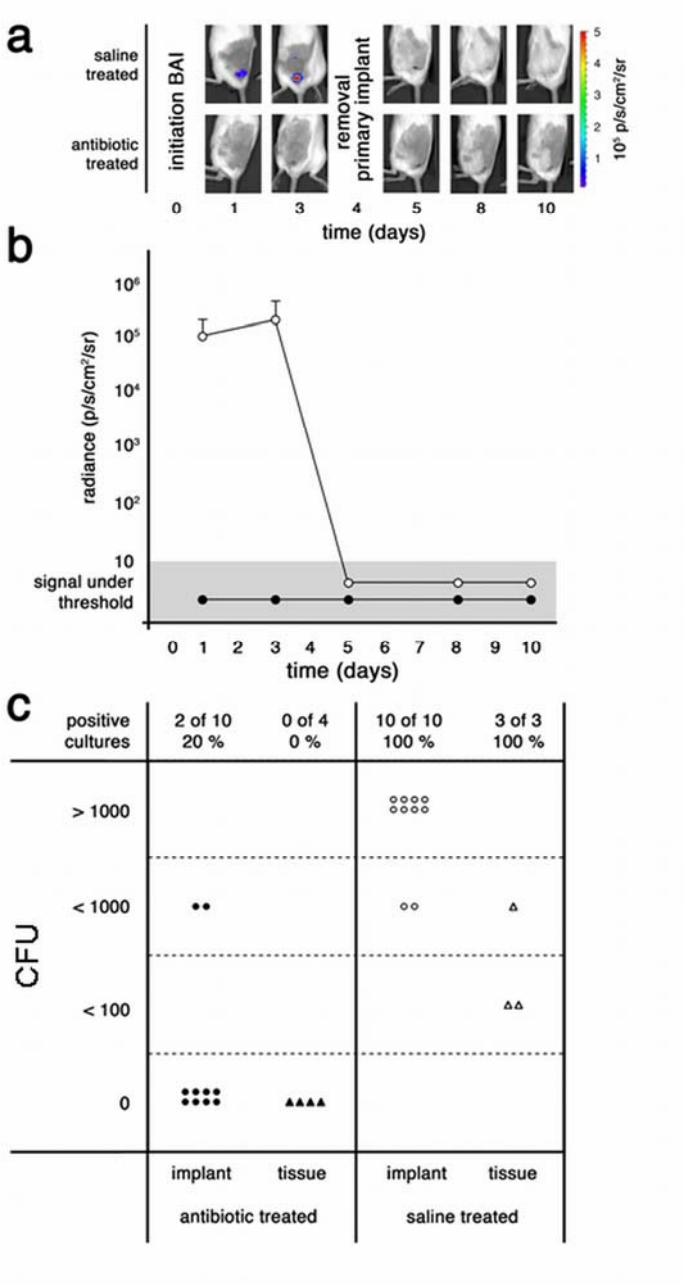


Figure 2. Infection of primary silicone rubber discs and surrounding tissue.
A) Examples of bioluminescent images projected on a grey-scale image of a representative mouse, with the time-point of removal of the primary discs indicated.
B) Mean bioluminescence (radiance p/s/cm^2/sr) in the antibiotic- and saline-treated groups with an implant present till day 4 and without an implant after day 4, presented as means ± SD over 10 mice (● antibiotic treated group; ○ saline treated group).
C) Numbers of colony forming units (CFU) as determined by plate counting from primary implants (● antibiotic treated group; ○ saline treated group), explanted at day 4, and tissue samples taken at day 10 (▲ antibiotic treated group; △ saline treated group). The difference between the saline and antibiotic treated group is significant for the silicone rubber disks ($p < 0.05$). Note that for tissue samples data are expressed per gram tissue (CFU g^{-1}). Since 6 out of the 10 mice received a secondary implant, tissue samples were only taken from 4 mice. Note that one tissue sample in the saline treated group was lost during processing.

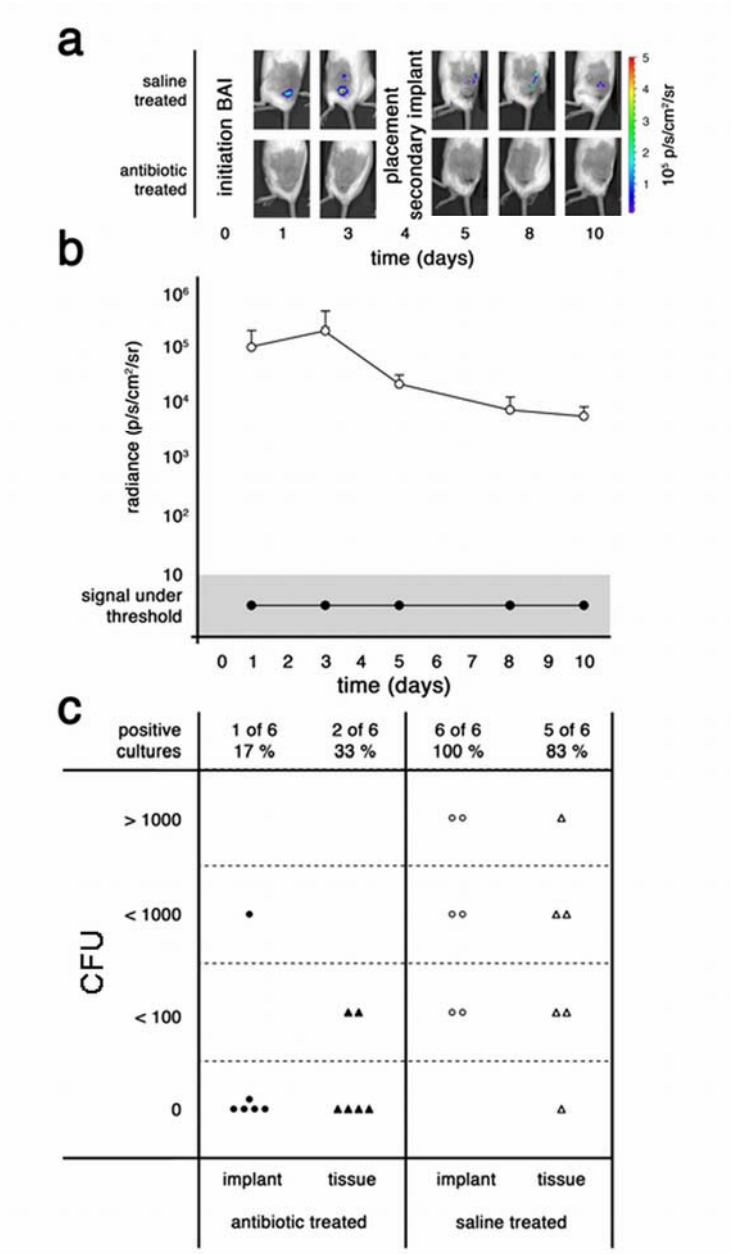


Figure 3. Infection of secondary silicone rubber discs and surrounding tissue.
A) Examples of bioluminescent images projected on a grey-scale image of a mouse, with the time-points of removal of the primary silicone rubber discs and insertion of secondary discs indicated.
B) Mean bioluminescence (radiance, p/s/cm^2/sr) in the antibiotic- and saline-treated groups, presented as means ± SD over 6 mice (● antibiotic treated group; ○ saline treated group).
C) Numbers of colony forming units (CFU) on agar plates from secondary implants (● antibiotic treated group; ○ saline treated group) and tissue samples (▲ antibiotic treated group; △ saline treated group), both collected at day 10, i.e. the end of the experimental period. The difference between the saline and antibiotic treated group is significant for the silicone rubber disks ($p < 0.05$). Note that for tissue samples taken at the end of the experimental period, data are expressed per gram tissue (CFU g^{-1}).

Plate counting showed that all secondary discs (which were implanted for 6 days) in saline-treated mice demonstrated bacteria and 83% of the surrounding tissue was positive for bacteria (see Figure 3C). However, despite the absence in tissue samples in the antibiotic-treated group without a secondary implant, bacteria were cultured from 17% of the secondary implanted discs in the antibiotic-treated group and from 33% of the surrounding tissue samples. The differences between the disks from the saline treated group and antibiotic treated group are significant, whereas the difference between the tissue samples is not significant.

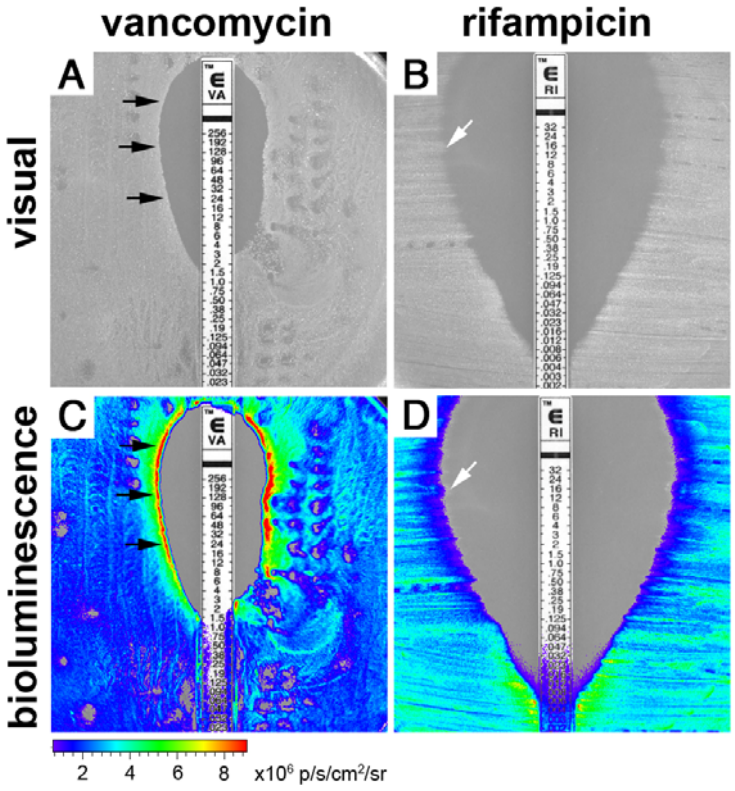


Figure 4. Regular light photographs (upper panel) and bioluminescence images (lower panel) of *S. aureus* Xen29 exposed to vancomycin (A and C) and rifampicin (B and D) in E-tests®. The white arrows show a reduction of bioluminescence as a result of exposure to rifampicin. In contrast, exposure of *S. aureus* Xen29 to vancomycin resulted in an increased bioluminescent signal (black arrows). The positions of the arrows in the pictures in the upper panel correspond with their positions in the lower panel.

MIC-values and S. aureus *Xen29 bioluminescence*

MIC-values of *S. aureus* Xen29 against vancomycin and rifampicin were 3 and 0.006 μg ml^{-1}, respectively (see Figures 4A and 4B). Interestingly, vancomycin induced an

increase in bioluminescence on the edge of the inhibition zone (Figure 4C, black arrows), whereas surprisingly rifampicin induced a decrease in bioluminescence at the transition from growth to no growth (Figure 4D, white arrows).

Discussion

Infection is a devastating complication in biomaterial implant surgery and results in considerable patient morbidity and need for revision surgery.[9,22,23] Following revision secondary implants are at even greater risk of becoming infected after BAI of a primary implant. This study indicates that one out of six implanted secondary silicone rubber discs becomes infected within 5 days after insertion despite antibiotic treatment and despite the observation that a sample of tissue from the infected primary implant site was devoid of viable bacteria at day 10. Moreover, not only the implant but also the tissue sample surrounding a secondary implant appeared infected in two out of six cases. In the absence of antibiotic treatment, all secondary silicone rubber discs (6/6) and nearly all (5/6) tissue samples became infected.

It is important to mention that in the antibiotic treated group secondary implanted silicone rubber discs became infected despite the fact that no bacteria could be retrieved from surrounding tissue samples. This clearly demonstrates the limitations of tissue sampling by itself. In daily clinical practice, it is known that a tissue sample taken from the neighborhood of an infected implant, which is negative for bacteria, is not always indicative for the absence of infection. Extensive microbiological analyses of explanted total hip arthroplasties indicated septic-loosening in 86% of all cases, while routine hospital culturing revealed infection in only 41%.[24] For this reason, it is advocated e.g. in orthopedics that multiple tissue samples should be taken to detect septic loosening in revision surgery.[24] The interstitial milieu surrounding prosthetic implants is known to represent a region of local immune depression,[25] which is susceptible to microbial colonization and thus highly favorable to (re-)infection.[26,27] In this niche, bacteria remain present in a metabolically less active state and in low numbers,[6,28] which decreases the sensitivity of microbiological evaluation (i.e. the detection of viable

bacteria) and efficacy of antibiotic treatment. Our results showed that removal of the primary implant without antibiotic therapy reduced the number of bacteria in the tissues dramatically, as demonstrated by bioluminescence, but did not result in aseptic cultures, leading to an almost 100% infection rate of both implant and surrounding tissue after 6 days. These findings correspond with current clinical experiences that a BAI is treated best with rigorous and long-term antibiotic therapy in combination with removal of the infected implant.

Bioluminescence has shown to be a reliable biomarker for the presence of viable bacteria, with a high correlation between the light signal and *ex vivo* bacterial counts.[15-17] However, with respect to the evaluation of secondary implant infection, especially after antibiotic treatment, its sensitivity requires further improvement. Bioluminescent signals were generally below the detection threshold, despite the fact that a significant bacterial presence was found on implants as well as in peri-implant tissues by *ex vivo* analyses. Possibly, bacterial presence was too low for detection by the IVIS, but it is also feasible that the *S. aureus* Xen29 were in a relatively low state of metabolic activity in the biofilm after antibiotic treatment, resulting in a weak bioluminescent signal.[29]

Imaging of *S. aureus* Xen29 bioluminescence in *ex vivo* E-test evaluations indicated that high doses of both vancomycin and rifampicin yielded unambiguous complete growth inhibition, accompanied by complete quenching of the bioluminescent signal. Interestingly however, around the antibiotic MIC it appears that vancomycin actually enhanced bioluminescence from *S. aureus* Xen29. Possibly sub-inhibitory concentrations of vancomycin cause an increased metabolic activity in the cell and as a consequence enhance bioluminescence. Thus, the bioluminescent signal as a result of the reporter system inserted in *S. aureus* Xen29[1,16-18] might not be stable during its growth in the presence of antibiotics. Earlier, it was demonstrated that also temperature changes or reduction of oxygen influence the bioluminescent signal.[16,17] It is unclear at present why vancomycin enhances bioluminescence, while rifampicin decreases bioluminescence of *S. aureus* Xen29 at the limit of their effective concentrations.

Conclusion

This study shows that there is an enhanced risk upon infection in biomaterials implant revision surgery due to BAI of a primary implant. Secondary discs became infected within days after revision surgery, even when no viable bacteria had been retrieved from tissue samples. This emphasizes the need for full clearance of the infection, also from surrounding tissues prior to implantation of a secondary implant. Based on the problematic experiences in revision surgery after BAI of various types of primary implants, this may require close collaboration between medical microbiologists and surgeons to ensure full clearance of the infection before revision surgery.

References

1. Engelsman AF, Van der Mei HC, Ploeg RJ, Busscher HJ. The phenomenon of infection with abdominal wall reconstruction. *Biomaterials* 2007; 28:2314-2327.
2. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; 284:1318-1322.
3. Monzón M, Oteiza C, Leiva J, Lamata M, Amorena B. Biofilm testing of *Staphylococcus epidermidis* clinical isolates: low performance of vancomycin in relation to other antibiotics. *Diagn Microbiol Infect Dis* 2002; 44:319-324.
4. Wilcox M, Kite P, Mills K, Sudgen S. *In situ* measurement of linezolid and vancomycin concentrations in intravascular catheter-associated biofilm. *J Antimicrob Chemother* 2001*;* 47:171-175.
5. Yao Y, Sturdevant D, Otto M. Genome wide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. *J Infect Dis* 2005; 191:289-298.
6. Broekhuizen CAN, De Boer L, Schipper K, Jones CD, Quadir S, Feldman RG Dankert J, Vandenbroucke-Grauls CMJE, Weening JJ, Zaat SAJ. Peri-implant tissue is an important niche for *Staphylococcus epidermidis* in experimental biomaterial-associated infection in mice. *Infect Immun* 2007; 75:1129-1136.
7. Boelens JJ, Dankert J, Murk JL, Weening JJ, Van der Poll T, Dingermans KP, Koole L, Laman JD, Zaat SAJ. Biomaterial-associated persistence of *Staphylococcus epidermidis* in pericatheter macrophages. *J Infect Dis* 2000; 181:1337-1349.

8. Broekhuizen CAN, de Boer L, Schipper K, Jones CD, Quadir S, Vandenbroucke-Grauls CMJE, Zaat SAJ. *Staphylococcus epidermidis* is cleared from bacterial implants but persists in peri-implant tissue in mice despite rifampicin/vancomycin treatment. *J Biomed Mater Res A* 2008; 85:498-505.

9. Elek SD, Conen PE. The virulence of *Staphylococcus pyogenes* for man; a study of the problems of wound infection. *Br J Exp Pathol* 1957; 38:573-586.

10. Lidwell OM, Lowbury EJ, Whyte W, Blowers R, Stanley SJ, Lowe D. Effect of ultraclean air in operating rooms on deep sepsis in the joint after total hip or knee replacement: a randomised study. *Br Med J* 1982; 285:10-14.

11. Zimmerli W, Waldvogel FA, Vaudaux P, Nydegger UE. Pathogenesis of foreign body infection: description and characteristics of an animal model. *J Infect Dis* 1982; 146:487-497.

12. Del Pozo JJ, Patel R. The challenge of treating biofilm-associated bacterial infections. *Clin Pharmacol Ther* 2007; 82:204-209.

13. Licht MR, Montague DK, Angermeier KW, Lakin MM. Cultures from genitourinary prostheses at reoperation: questioning the role of *Staphylococcus epidermidis* in periprosthetic infection. *J Urol* 1995; 154:387-390.

14. Campoccia D, Montanaro L, Arciola CR. The significance of infection related to orthopedic devices and issues of antibiotic resistance. *Biomaterials* 2006; 27:2331-2339.

15. Engelsman AF, Van der Mei HC, Francis KP, Busscher HJ, Ploeg RJ, Van Dam GM. Real time, non-invasive monitoring of bacterial presence in a soft tissue implant infection model. *J Biomed Mater Res B* 2009; 88:123-129.

16. Kadurugamuwa JL, Sin L, Albert E, Francis KP, DeBoer M, Rubin M, Bellinger-Kawahara C, Parr Jr. TR, Contag PR. Direct continuous method for monitoring biofilm infection in a mouse model. *Infect Immun* 2003; 71:882-890.

17. Kadurugamuwa JL, Sin LV, Yu J, Francis KP, Kimura R, Purchio TF, Contag PR. Rapid direct method for monitoring antibiotics in a mouse model of bacterial biofilm infection. *Antimicrob Agents Chemother* 2003; 47:3130-3137.

18. Monzón M, Garcia-Alvarez F, Lacleriga A, Gracia E, Leiva J, Oteiza C, Amorena B. A simple infection model using pre-colonized implants to reproduce rat chronic *Staphylococcus aureus* osteomyelitis and study antibiotic treatment. *J Orthop Res* 2001; 19:820-826.

19. Francis KP, Joh D, Bellinger-Kawahara C, Hawkinson MJ, Purchio TF, Contag PR. Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel luxABCDE construct. *Infect Immun* 2000; 68:3594-3600.

20. Xiong YQ, Willard J, Kadurugamuwa JL, Yu J, Francis KP, Bayer AS. Real-time *in vivo* bioluminescent imaging for evaluating the efficacy of antibiotics in a rat Staphylococcus aureus endocarditis model. Antimicrob *Agents Chemother* 2005; 49:380-387.

21. Yu J, Wu J, Francis KP, Purchio TF, Kadurugamuwa JL. Monitoring *in viv*o fitness of rifampicin-resistant *Staphylococcus aureu*s mutants in a mouse biofilm infection model. *J Antimicrob Chemother* 2005; 55:528-534.

22. Cyrochristos DJ, Papadopoulos O; Liapis C; Felekonras EL, Giannopoulos AM, Bastounis E. Coverage strategies in exposed implants. *Am Surg* 2009; 75:1132-1138.

23. Greenberg JJ. 2010. Can infected composite mesh be salvaged? *Hernia* DOI 10.1007/s10029-010-0694-8.

24. Neut D, Van Horn JR, Van Kooten TG, Van der Mei HC, Busscher HJ. Detection of biomaterial-associated infections in orthopeadic joint implants. *Clin Orthop* 2003; 413:261-268.

25. Gristina AG. Implant failure and the immuno-incompetent fibro-inflammatory zone. *Clin Orthop Relat Res* 1994; 298:106-118.

26. Stoppa R. About biomaterials and how they work in groin hernia repairs. *Hernia* 2003; 7:57-60.

27. Schierholz JM, Beuth JJ. Implant infections: a haven for opportunistic bacteria. *J Hosp Infect* 2001; 49:87-93.

28. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 2001; 9:34-39.

29. Sjollema J, Sharma PK, Dijkstra RJB, Van Dam GM, Van der Mei HC, Engelsman AF, Busscher HJ. The potential for bio-optical imaging of biomaterial-associated infection *in vivo*. *Biomaterials* 2010; 31:1984-1995.

Chapter 3

The Inhibition of the Adhesion of Clinically Isolated Bacterial Strains on Multi-Component Crosslinked Poly(ethylene glycol)-Based Polymer Coatings

Reproduced with permission of Elsevier from: Isabel C. Saldarriaga Fernández, Henny C. van der Mei, Michael J. Lochhead, David W. Grainger, Henk J. Busscher. *Biomaterials* **2007**, 28: 4105-4112.

Abstract

This study examined bacterial adhesion to a new multi-component crosslinked poly(ethylene glycol)-based polymer coating that can be applied by spin coating or spraying onto diverse biomaterials. Five clinically isolated bacterial strains involved in biomaterial-centred infections were studied in a well-characterized parallel-plate flow chamber at different shear rates and after exposure of the coating to different physiological fluids. The new chemistry inhibits non-specific biomolecular and cell binding interactions. Relative to glass, the coating reduced adhesion of all strains used in this study by more than 80%, with exception of *Escherichia coli* O2K2. Reductions in adhesion of *Staphylococcus epidermidis* 3399 persisted beyond 168 h exposure of the coatings to PBS or urine, but not after exposure to protein-rich fluids as saliva and blood plasma, despite evidence from X-ray photoelectron spectroscopy indicating that coating integrity was not affected by exposure to these fluids. We conclude that this new coating chemistry provides beneficial properties to prevent or hinder bacterial adhesion and colonization in applications where low protein-conditions prevail.

Introduction

Biomaterial-associated infections are generally of low incidence but due to extensive significance (high device and patient numbers) and increasing complications (i.e. antibiotic resistance) across all device categories, these infections represent a substantial total clinical caseload annually. Associated high health care cost burdens for infection mitigation, patient discomfort and not infrequently, death, present motivation to provide new solutions to this problem.[1]

Bacterial adhesion is a critical step in the pathogenesis of a biomaterial-centred infection and eventually leads to the formation of a biofilm. Pathogens in a biofilm are encased in a slime layer that protects these organisms from host immune defences and clinical antibiotics. Hence, infections are difficult to eradicate and removal of an infected implant is often the only remedy. Therefore, in order to avoid infectious complications with implants, surfaces and coatings non-adhesive to bacteria are essential. Over the past, coatings with altered surface charge or hydrophobicity have been developed that discourage non-specific bacterial adhesion and have been advocated for different clinical applications.[2] Specific bacterial adhesion can be discouraged by application of adsorbed proteinaceous coatings, as shown with adsorbed albumin.[3-5] Poly(ethylene glycol) (PEG) coatings have been extensively studied as a method to prevent protein adsorption, bacterial adhesion, and biomaterial-centred infections.[6-8] Tethered PEG brush-like coating configurations form a hydrated, steric barrier, repelling micro-organisms and proteins approaching the surface.[8] In general, PEG coatings have been shown to reduce adhesion of bacteria and yeast *in vitro*, but after exposure to physiological fluids *in vitro* or *in vivo*, reductions in bacterial adhesion are usually small or even lost.[9] This performance degradation has most often been attributed to eventual overwhelming of the surface by continuous bulk protein assault, or coating degradation (e.g. hydrolysis, chain cleavage, surface removal). Surprisingly, to date, despite their popularity in the academic literature, few biomedical commercially marketed coatings based on PEG are available, perhaps due to difficulties in creating surface-bound thin films amenable to industrial scale processing and properties.

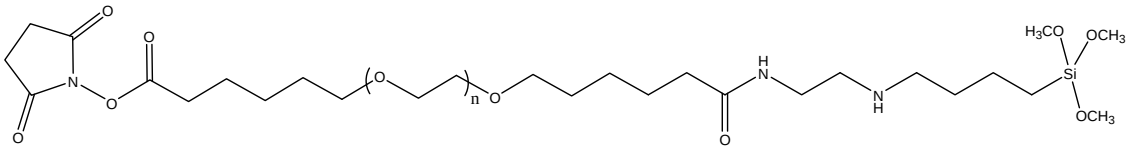
A new commercial multi-component crosslinked PEG-based polymer coating (OptiChem®, Accelr8 Technology Corporation. Denver, CO) has been recently developed to inhibit non-specific biomolecular adsorption, protein and cell binding. Composed of three core coating components applied to surfaces from a volatile carrier solvent, the chemistry is readily applied in a single step and is compatible with diverse substrates, including glass, metal oxides, and numerous plastics. The components crosslink into a conformal, robust optically transparent thin film, with functional coupling chemistry and bio-immobilization capabilities demonstrating substantial practical utility in commercial microarray diagnostics and selective cell adhesion studies.[10] Anti-bacterial properties are also interesting. Hence, the effectiveness of this multi-component crosslinked PEG-based polymer coating against adhesion of different clinically isolated bacterial strains involved in biomaterial-associated infections, including *Staphylococcus epidermidis* 3399, *Staphylococcus epidermidis* HBH 276, *Streptococcus salivarius* GB24/9, *Escherichia coli* O2K2 and *Pseudomonas aeruginosa* #3 were assessed. Furthermore, coating stability after exposure to different physiological fluids is reported. Coating chemistry was characterized by X-ray photoelectron spectroscopy (XPS), streaming potential and water contact angle measurements.

Materials and methods

Multi-component crosslinked PEG-based polymer coating

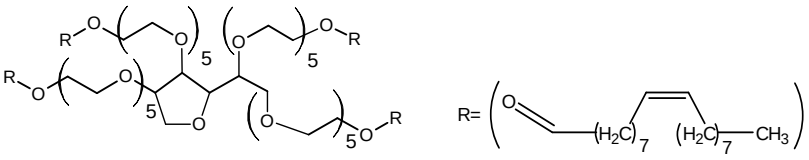
The chemistry and coating properties of the PEG-based polymer coating have been previously reported in substantial detail.[10] Briefly, the chemistry comprises three primary coating matrix components mixed into a volatile carrier solvent. The first component ("active component") is a hetero-bifunctional PEG molecule (M_w 3400) terminated with a succinimidyl ester (NHS) serving as functional group in the final coating, and an alkoxysilane terminus that functions as a reactive crosslinking group, providing covalent attachment within the coating matrix and to certain substrates. The second component is the "matrix-forming component" a non-ionic surfactant containing ethylene oxide repeat units (polyoxyethylene sorbitan tetraoleate).

a. Active PEG heterobifunctional base component



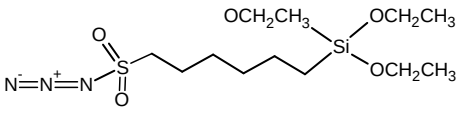
A-Poly(ethylene glycol)-B

b. Matrix-forming ethylene glycol oligomer component



Polyoxyethylene sorbitan tetraoleate

c. Molecular cross-linking component



6-azidosulfonylhexyltriethoxy silane

a + b + c

Application by carrier solvent onto the substrate by spin-coat, solvent removal and thermal cure

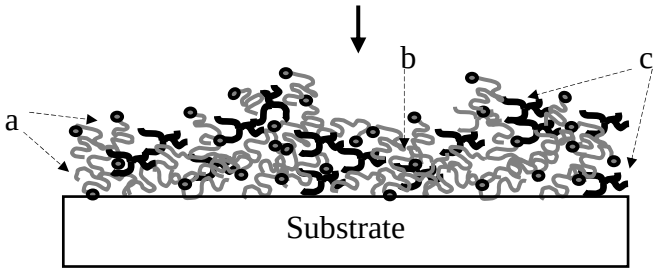


Figure 1. OptiChem® coating components (a) active component, (b) molecular crosslinking structure component and (c) matrix forming component, and schematic process.

The third component is the intermolecular cross-linking component, azidosilane. Upon thermal activation after coating, the azido group inserts into aliphatic or aromatic bonds within the coating matrix or on organic substrates; the silane end crosslinks with other silanes in the matrix and provides covalent linkage to surface oxides on certain

substrates. A complete schematic reaction between the three coating components is presented in Figure 1, yielding a robust, PEG-grafted surface with numerous functional group capabilities. This single-step crosslinked PEG–based coating formulation attaches covalently upon curing to surfaces and can be reproducibly applied with conventional industrial techniques, such as spin coating, spraying, dip-coating, and other methods.

The multi-component crosslinked PEG-based polymer coating was applied by spin coating from dimethyl sulfoxide (DMSO) onto borosilicate glass microscope slides (Schott Glass, D263, 75.6 x 25.0 x 1.0 mm) first cleaned using a 60°C alkaline detergent with sonication, rinsed extensively with water, racked, dried by centrifugation, and stored in a clean room dry box until coating (within 24 h after cleaning). The coating was cured thermally at 100°C under vacuum (0.1 mm Hg pressure) to drive the cross-linking reaction within the film, and rinsed briefly with ultrapure water to remove any loosely bound material, immediately dried in ambient air in a centrifuge and stored dry in sealed moisture barrier bags with desiccant. The film final thickness is approximately 10 - 20 nm (on glass) as determined by spectroscopic ellipsometry, and after hydration, the film expands significantly to thicknesses of between 50 and 100 nm.[11]

These coatings provide a PEG-tethered NHS reactivity after cure to allow specific attachment of certain nucleophilic molecules (e.g. reactive amines, see Figure 1). For adhesion studies, reactive NHS was deactivated (e.g. eliminating the reactive end groups) by submerging coated slides in methoxyethylamine and borate buffer. The primary amine terminus reacts with the coating NHS groups to create amide-linked ethyl methoxy groups terminating the crosslinked PEG chains.

Characterization of bacterial strains and multi-component crosslinked PEG-based polymer coatings

Contact angles. The hydrophobicities of both the coatings and bacterial strains were measured by advancing-type water contact angles (θ_w) at room temperature (25°C) using the sessile drop technique with a home-made contour monitor. To measure bacterial contact angles, bacteria cultured in growth media were first harvested by centrifugation, washed twice with demineralized water and finally resuspended in demineralized water. Bacteria were deposited in layers onto a 0.45µm pore size HA

membrane filter (Millipore Corporation, Bedford, MA, USA) using negative pressure. The filters containing the bacteria were placed on a metal disc and allowed to air-dry until plateau contact angles could be measured. Three filters were prepared from one bacterial culture and six droplets were placed at different spots on each filtered lawn of bacteria. Water contact angles were measured in triplicate on different coated slides and also for three different bacterial cultures.

X-ray photoelectron spectroscopy (XPS). X-ray photoelectron spectroscopy (XPS) was performed using an S-probe spectrometer (Surface Science Instruments, Mountain View, CA, USA) with X-rays (10kV, 22mA, spot size of 250 x 1000 µm) sourced from an aluminium anode. The analyzer was placed at a 35° take off angle (i.e. the angle between the surface plane and the axis of the analyzer lens), yielding a sampling depth of ~15 nm. Broad spectrum survey scans (binding energy range of 1 to 1100eV) were made at low resolution (pass energy, 150 eV), and peaks over a 20-eV binding energy range were recorded at high resolution (pass energy, 50 eV) for C1s, O1s, N1s and Si2s. The area under each peak was used to calculate peak intensities, yielding elemental surface concentrations for carbon, oxygen, nitrogen and silicon, after correction with sensitivity factors provided by the manufacturer. The elemental surface composition of the OptiChem® coating was expressed in atomic percentage (%), setting %C + %O + %N + %Si to 100%. Results are the average of measurements performed on at least two spots of a single sample.

Streaming potentials. Streaming potentials were measured in phosphate buffered saline (PBS, pH 6.8) in a home-made parallel plate flow chamber employing rectangular platinum electrodes (5.0 mm x 25.0 mm) located at both ends of the flow chamber. Streaming potentials at 10 different pressures ranging from 37.5 to 150 Torr were measured, each pressure applied for 10 s in both directions. Three independent measurements were made with a new coated glass slide used for each measurement.

*Bacterial zeta potentials***.** Bacterial zeta potentials *(ζ)* were calculated from the electrophoretic mobilities of the different bacterial strains measured with a Lazer Zee

Meter 501 (PenKem Inc., Bedford Hills, NY). Bacterial strains were harvested and washed as described above for contact angle analysis, and resuspended in PBS at pH 6.8. Electrophoretic mobilities were measured from at least 100 bacteria using a tracking image analysis system and converted to zeta potentials by applying the Helmholtz-Smoluchowski equation. Three separate bacterial cultures of each strain were used.

Bacterial adhesion

Bacterial strains and growth conditions. Five different bacterial strains, all clinical isolates, were used, including *S. epidermidis* 3399, *S. epidermidis* HBH 276, *S. salivarius* GB 24/9, *P. aeruginosa* #3 and *E. coli* O2K2. The strains were first grown from a frozen stock on blood agar plates by incubation during 24 h at 37°C in ambient air. These plates were kept at 4°C. Several colonies were used to inoculate 10 ml of Todd Hewitt Broth (THB, OXOID, Basingstoke, UK) for *S. salivarius* and 10 ml of tryptone soya broth (TSB, OXOID) for the other strains. These precultures were incubated for 24 h at 37°C and used to inoculate second cultures of 200 ml TSB or THB, the latter being allowed to grow overnight (16 h) at 37°C.

Bacteria from the second cultures were harvested by centrifugation (5 min at 5000 g at 10°C for staphylococci and 5 min at 10000 g at 10°C for the other strains) and washed twice with demineralized water. Bacteria were sonicated intermittently on ice (20 s) to break bacterial chains and aggregates and obtain single cells. Bacteria were then resuspended in 200 ml PBS, to a concentration of 3 x 10^8 bacteria ml^{-1}.

Parallel plate flow chamber and image analysis. Microbial adhesion and detachment from OptiChem®-coated slides under laminar flow was directly assessed at room temperature using real- time (*in situ*) image analysis in a parallel plate flow chamber (175 mm length x 17 mm width x 0.75 mm depth) as described in detail elsewhere.[12] Before each experiment, PBS was flowed through the system to remove all bubbles from the tubing and flow chamber, after which flow was switched to a bacterial suspension that circulated through the system during 4 h at four increasing flow rates i.e. known shear rates. Flow rates (Q) decreased per hour from 0.117 ml s^{-1} to 0.025 ml s^{-1}, 0.008 ml

s^{-1} and 0.003 ml s^{-1} in the fourth hour. These flow rates correspond to wall shear rates (σ) of 73, 16, 5 and 2 s^{-1} as calculated from[12]

$$\sigma = \frac{3Q}{2(h/2)^2 w}$$

where, h is the height and w the width of the flow chamber. Studies proceeded in descending order of flow rates, because high flow rates clearly produce high wall shear rates that prevent deposition of bacteria or detach already adherent bacteria.[12,13] Accordingly, results obtained at a lower shear rate are not significantly influenced by the results obtained at the higher shear rate.

During bacterial deposition, images were taken from the bottom plate, consisting of the coated glass slide. The top plate of the chamber was a bare glass slide cleaned in 2% RBS 35 (Omnilabo International BV, Breda, The Netherlands) detergent solution under sonication, thoroughly rinsed with water, cleaned with methanol and washed with demineralized water to remove any impurities present on the surface. All bacterial adhesion data on coated glass were compared with data for bare glass.

To obtain images at each shear rate, five images were taken at the end of every hour until completion of the fourth hour. Subsequently, to assess the strength of bacterial adhesion, an air bubble was passed through the chamber, producing detachment forces measured on an adhering micron-sized particle to be approximately 1 x 10^{-7} N.[12] Then, the suspension was switched again to a buffer solution (PBS) and five final images were taken. All experiments were carried out at least three times with separately grown micro-organisms and new coated glass slides.

Effectiveness and stability of the multicomponent crosslinked PEG-based polymer coating in physiological fluids

In order to determine the stability of OptiChem® coatings, coated glass slides were exposed to 30 ml of PBS, pooled human urine, pooled human full blood plasma or pooled human whole saliva for 24, 48 or 168 h at room temperature. Bare glass was included as a control. Coated glass slides were taken out of the fluids after the designated time intervals, rinsed briefly with demineralized water and their effectiveness assessed by

evaluating the adhesion of *S. epidermidis* 3399, as described above. Chemical changes occurring during exposure to the biological fluids were determined using XPS. Stability experiments were carried out in single fold.

Statistical analysis

To analyze differences between bacterial adhesion to glass and OptiChem® coatings, statistically significant differences ($p<0.05$) between the means of the two groups were determined by the two-tailed Student's t-test.

Results

Physicochemical characterization of the bacterial strains and the multicomponent crosslinked PEG-based coating

The zeta potentials (ζ) and the water contact angles (θ_w) measured on the bacterial lawns used in this study are listed in Table 1. The cell surfaces of all strains were negatively charged and hydrophilic.

Table 1. Water contact angles and zeta potentials for *S. epidermidis* 3399, *S. epidermidis* HBH 276, *S. salivarius* GB24/9, *E. coli* O2K2 and *P. aeruginosa* # 3, as well as their percentage air bubble-induced detachment from glass and OptiChem® coatings. ± signs represent the average standard deviation over three separate experiments with separately cultured bacteria and new (coated) glass slides.

Bacterial Strain	ζ (mV)	θ_w (degrees)	Detachment from glass (%)	Detachment from OptiChem® (%)
S. epidermidis 3399	-15 ± 3	30 ± 3	6 ± 3	87 ± 8
S. epidermidis HBH 276	-11 ± 2	34 ± 6	7 ± 6	82 ± 15
S. salivarius GB24/9	-18 ± 1	22 ± 5	34 ± 9	90 ± 4
P. aeruginosa #3	-8 ± 3	19 ± 1	32 ± 26	91 ± 4
E. coli O2K2	-18 ± 6	14 ± 2	83 ± 2	65 ± 11

Contact angles varied between 14 ± 2 degrees for *E. coli* O2K2 to 34 ± 6 degrees for *S. epidermidis* HBH 276. The PEG-based polymer coating also exhibited a negatively charged and hydrophilic surface with a zeta potential of -10 ± 1 mV and a water contact angle of 39 ± 1 degrees.

Table 2. Percentage elemental composition of OptiChem® coatings prior to and after exposure to different physiological fluids for 24, 48 or 168 h, setting %C + %O + %N + %Si to 100%, as well as the percentage of air bubble induced detachment of *S. epidermidis* 3399 . The elemental composition of glass is also given for reference purposes. ± signs in XPS data represent the average standard deviation over two separate measurements, while adhesion experiments were performed once for the purpose of demonstrating coating stability.

		%C	%O	%N	%Si	Detachment (%)
	Glass	25.4 ± 0.7	43.9 ± 0.1	1.6 ± 0.2	17.5 ± 0.2	6
	OptiChem®	55.7 ± 0.5	36.2 ± 0.7	1.7 ± 0.2	6.5 ± 0.0	87

Bathing fluid	**Time (h)**	**OptiChem® after exposure**				**Detachment (%)**	
		%C	**%O**	**%N**	**%Si**	**Glass**	**OptiChem®**
PBS	24	36.7 ± 0.0	43.2 ± 0.0	0.0 ± 0.0	18.3 ± 0.0	6	12
	48	59.9 ± 1.3	30.3 ± 0.8	2.5 ± 0.2	6.9 ± 0.3	22	58
	168	51.4 ± 0.2	36.2 ± 0.6	1.6 ± 0.2	8.6 ± 1.1	20	81
Urine	24	53.3 ± 0.3	36.5 ± 0.2	1.1 ± 1.6	8.3 ± 0.0	11	72
	48	53.4 ± 1.7	35.4 ± 1.5	2.5 ± 0.1	8.3 ± 0.3	51	90
	168	65.5 ± 1.9	24.5 ± 0.4	3.8 ± 0.2	4.3 ± 0.8	15	42
Plasma	24	65.6 ± 0.2	19.2 ± 0.3	13.5 ± 0.0	1.1 ± 0.2	79	76
	48	69.2 ± 1.4	17.1 ± 0.4	13.1 ± 0.1	0.0 ± 0.0	55	95
	168	70.5 ± 2.3	17.0 ± 1.3	10.0 ± 0.5	2.5 ± 0.5	61	93
Saliva	24	61.2 ± 0.0	24.7 ± 0.0	10.7 ± 0.0	3.4 ± 0.0	69	70
	48	61.0 ± 0.0	25.1 ± 0.0	10.3 ± 0.0	3.5 ± 0.0	44	92

Coating application to glass decreased the surface concentration of oxygen and silicon, while increasing the surface concentration of carbon. The surface concentration of nitrogen remained relatively constant (Table 2).

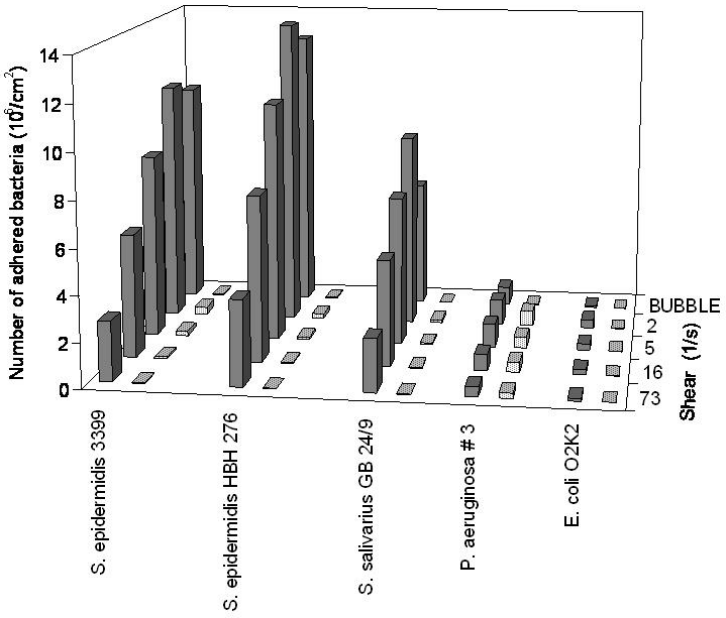


Figure 2. Adhesion of *S. epidermidis* 3399, *S. epidermidis* HBH 276, *S. salivarius* GB24/9, *P. aeruginosa* # 3 and *E. coli* O2K2 to glass (black) and OptiChem® coatings (grey) as a function of wall shear rate. The initial shear rate of 73 s^{-1} was reduced stepwise after each hour. Bubble data corresponds to the retention of bacteria after the passage of an air bubble through the flow chamber at the end of an experiment.

Effectiveness and stability of the PEG-based coating in physiological fluids

Adhesion of *S. epidermidis* 3399 to glass and to the multicomponent crosslinked PEG-based polymer coatings previously exposed for different time intervals to PBS, human urine, human blood plasma and human saliva is presented in Figure 3. For comparison, adhesion of *S. epidermidis* 3399 to glass and to the coatings not exposed to physiological fluids (control) is also shown. Detachment percentages of *S. epidermidis* from glass and from the coatings exposed to PBS, urine, blood and saliva at all time intervals are presented in Table 2. The multicomponent crosslinked PEG-based polymer coatings did not show any considerable changes in staphylococcal adhesion after exposure to PBS for 168 h. Furthermore, adhesion to the coatings was always lower than adhesion to glass under the same conditions, and air bubble induced detachment was always higher (see Table 2). Exposure to urine for 24, 48 and 168 h yielded an increase in staphylococcal

adhesion on the coatings compared to PBS, but adhesion generally remained less than on glass. Moreover, bacteria detached, at all time intervals, more readily from the coating. A slight increase in the number of adhering staphylococci was observed for the coatings upon exposure to blood plasma, but air bubble induced detachment was generally larger on this surface than on glass (see Table 2).

After 24 h in saliva, a small increase in staphylococcal adhesion on the coatings was noticed at lower shear rates, but after 48 h adhesion decreased to baseline values, still no major differences in adhesion to glass and to OptiChem® coatings were found. Interestingly, throughout all experiments, adhesion of *S. epidermidis* 3399 to OptiChem® coatings remained less than 2 x 10^6 per cm^2, generally lower than on glass (0.4 x 10^6 to 10.6 x 10^6 per cm^2).

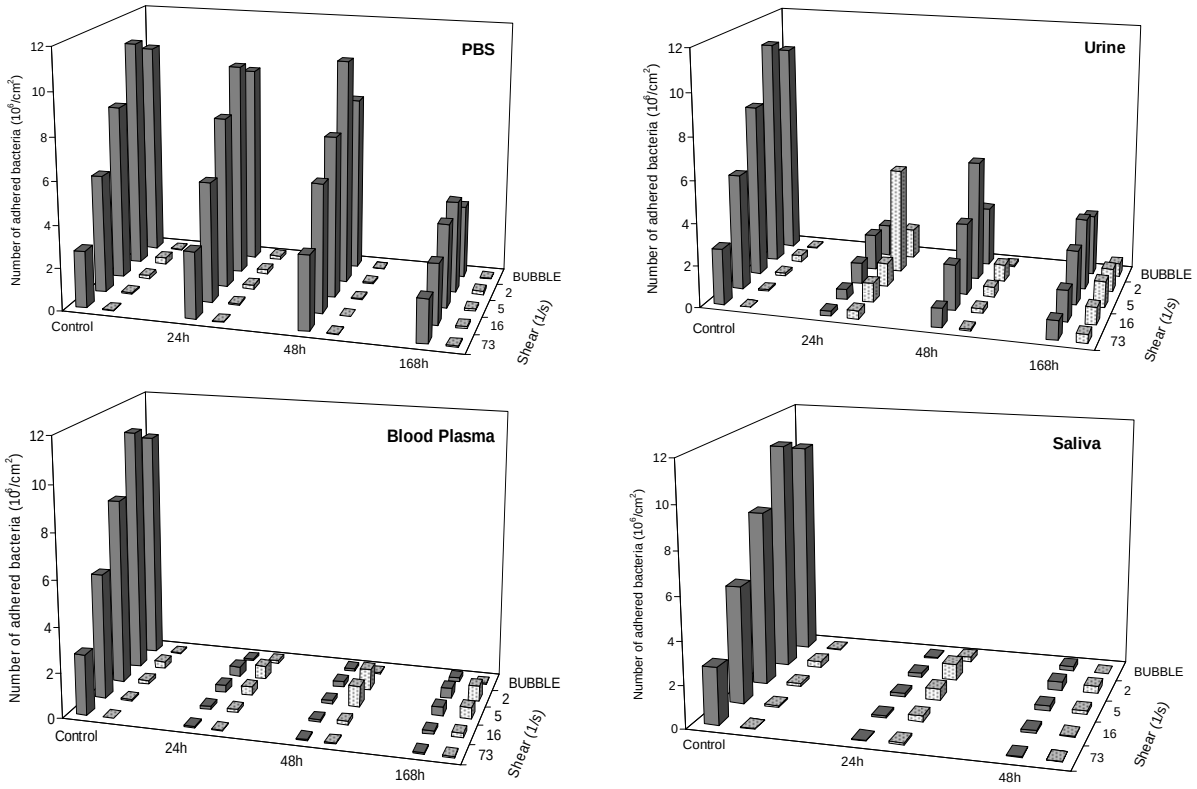


Figure 3. *S. epidermidis* 3399 adhering to glass (black) and OptiChem® coatings (grey) after exposure to PBS, urine, to blood plasma and saliva for 24, 48 and 168 h as function of the wall shear rate.

XPS was used to determine whether the coatings were chemically stable in PBS, urine, blood plasma and saliva. In Table 2, the chemical composition of OptiChem® coatings as measured by XPS after exposure to the different fluids are presented. Exposure of the

polymer coating to PBS and urine up to 168 h produced no major chemical changes in coating composition as ascertained by XPS elemental analysis. Coatings exposed to blood plasma and saliva, presented increased amounts of nitrogen and carbon, at the expense of silicon. Amount of surface elemental oxygen were not affected.

Discussion

The development of biomaterial surfaces less prone to infections has been a central medical device goal for decades. Different strategies have been investigated, most of them aimed at inhibiting bacterial adhesion and surface growth required for biofilm formation. So far, none of these approaches fully prevent bacterial adhesion either *in vivo* or *in vitro*,[2,14] although numbers of adherent cells can be significantly reduced, but primarily from non-physiological media. In addition, a relatively small number of reports describe effects of physiological fluids on the efficacy and the chemical stability of modified surfaces. The present study examines coating resistance to bacterial strains normally found in human skin, oral cavity, gastro-intestinal and urinary tract and major causes of implant and foreign body associated infections.[2,14,15] Importantly, coating stability in actual human-derived physiological fluids was also evaluated, providing an important assessment of relevance for these assays. These crosslinked PEG-based polymer coatings contributed to a significant reduction in adhesion of *S. epidermidis* 3399, *S. epidermidis* HBH 276, *S. salivarius* GB24/9, *E. coli* O2K2 and, to a lesser extent, of *P. aeruginosa* #3 in PBS when compared to bare glass. Additionally, these coatings contributed to a weaker bacterial binding than on glass. However, depending on the physiological bathing fluid, the coating effectiveness against adhesion can be notably altered.

Many different PEG-based or ethylene glycol-rich surfaces have been employed against biofilm formation, producing significant reductions in bacterial adhesion *in vitro* (up to 80%).[7,16-21] These coatings usually require multiple steps and reactions to apply, or surface modification, i.e. alkylsilane treatments, or use of other bonding promoters, or limitations to specific surface chemistries for the PEG coating immobilization. Thus, the

single-step formulation and surface chemistry-independence of OptiChem® coatings[10] represent advantages over other PEG-based coatings. Anti-adhesive and non-fouling PEG properties have been attributed to its high hydration capacity and stability, making surface adsorption by proteins or bacteria thermodynamically difficult. Binding leads to a repulsive osmotic interaction, making the adsorption and adhesion process weak or unfavourable.[2] Results from this study showed that adhesion to glass was higher when the cell surface was more hydrophobic, while adhesion to the coatings was higher when the bacterial surface charge was less negatively charged. This suggests that bacterial adhesion to the polymer coating is dominated by electrostatic interactions whereas adhesion to glass is dominated by hydrophobic interactions. According to this, an explanation for the observed increased affinity of *P. aeruginosa* for the polymer coating is its less negative bacterial surface charge. However, the relatively low effect of the OptiChem® coating in inhibiting *P. aeruginosa* adhesion is consistent with other surface modification studies.[22,23] *P. aeruginosa* # 3, classified as an adhesive strain, releases surface-active exopolymeric substances that can penetrate the PEO coating matrix, reducing PEO interfacial properties and increasing attractive interactions between bacteria and the coatings.[22]

Although the influence of shear on microbial adhesion to PEO brushes has been reported,[13] comparisons between the effectiveness of less-organized, crosslinked PEO-based polymer coatings and PEO brushes are limited. Additionally, the methodology employed here to assess shear-based bacterial adhesion is slightly different from that already reported for PEO brushes.[13] Here, we evaluated the change in numbers of surface-adherent bacteria per cm^2 upon hourly decrements of applied shear rate, while previous work analyzed changes in deposition rates upon 30 min changes in shear rates. Despite the differences in methodology, both studies demonstrate that bacterial adhesion to PEG coatings strongly decreases with increasing shear because adhesion forces to both PEG coatings are weak.

The influence of surface exposure to various human body fluids on PEG coatings after prolonged exposure has been previously investigated by others.[9,24,25] Contrary to reports for other PEG-based coatings,[9] XPS results indicate that crosslinked OptiChem® polymer coatings remained stable and effective against bacterial adhesion after 168 h

exposure in both urine and PBS. Little change in surface composition is observed over time. On the other hand, when the bathing fluid was human saliva, the anti-adhesive microbial activity of the coatings diminished. This has also been shown in other studies.[9,24] PEG brush configurations exposed to saliva had a reduced effectiveness against adhesion of a variety of bacterial strains,[9] attributed to mucins present in saliva.[24] Mucins bind to PEO surfaces and penetrate between the polymer chains, covering the coating surface and reducing its long-term effectiveness against bacteria. Another possibility frequently reported but seldom shown is coating degradation under human fluid exposure. OptiChem® degradation would have exposed the glass substratum surface beneath the coating, a feature detectable by XPS interrogation of silicon. Our XPS findings of decreased silicon signal from OptiChem® over time makes this option unlikely. A third explanation for changes in surface characteristics over time could be the formation of an adsorbed protein layer with time. Proteins contain both carbon and nitrogen contributing to increases in XPS-measured amounts of carbon and nitrogen on the coating over time in these fluids. Samples exposed to both saliva and blood plasma for 24 h showed reductions in surface anti-adhesive properties and increased nitrogen and carbon signals, consistent with reduced antimicrobial properties of PEG coatings in the presence of adsorbed plasma proteins found elsewhere.[25] The 24 h-exposure to blood plasma or saliva produces sufficient protein adsorption and formation of a conditioning film on OptiChem® rather than coating degradation and removal. This was confirmed by calculating adsorbed protein layer thickness (data not shown) based on XPS information showing that at all time intervals the chemical components of OptiChem® coatings remained stable and did not degrade.

Conclusions

A new commercial multi-component crosslinked PEG-based polymer coating (OptiChem®) strongly reduces adhesion of several clinically isolated bacterial strains *in vitro* from various physiological fluids and buffer by reducing the bacterial binding forces. The coating remains stable for over a week in these fluids. Best results in

inhibiting bacterial adhesion were found for PBS buffer and urine. However, the non-adhesive effectiveness of the coating in protein-rich physiological fluids (saliva or blood plasma) as opposed to PBS or urine decreases over time.

References

1. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* 1987; 237:1588-1595.
2. Roosjen A, Norde W, Van der Mei HC, Busscher HJ. The use of positively charged or low surface free energy coatings versus polymer brushes in controlling biofilm formation. *Progress Coll Polym Sci* 2006; 132:138-144.
3. An YH, Friedman RJ, Draughn RA, Smith EA, Nicholson JH, John JF. Rapid quantification of staphylococci adhered to titanium surfaces using image analyzed epifluorescence microscopy. *J Microbiol Methods* 1995; 24:37-40.
4. Kinnari TJ, Peltonen LI, Kuusela P, Kivilahti J, Kononen M, Jero J. Bacterial adherence to titanium surface coated with human serum albumin. *Otol Neurol* 2005; 26:380-384.
5. An YH, Bradley J, Powers DL, Friedman RJ. The prevention of prosthetic infection using a cross linked albumin coating in a rabbit model. *J Bone Joint Surg* 1997; 79-B:816-19.
6. Ratner BD, Hoffman AS, Schoen FJ, Lemons JE. Biomaterials Science. Elsevier Academic Press 2004; Second edition. Pages, editor
7. Roosjen A, Kaper HJ, Van der Mei HC, Norde W, Busscher HJ. Inhibition of adhesion of yeasts and bacteria by poly(ethylene oxide) brushes on glass in a parallel plate flow chamber. *Microbiol* 2003; 149:3239-3246.
8. Roosjen A, De Vries J, Van der Mei HC, Norde W, Busscher HJ. Stability and effectiveness against bacterial adhesion of poly(ethylene oxide) coatings in biological fluids. *J Biomed Mater Res Part B: Appl Biomat* 2005; 73:347-354.
9. Currie EP, Norde W, Cohen SMA. Tethered polymer chains: surface chemistry and their impact on colloidal and surface properties. *Adv Coll Interf Sci* 2003;100:205-265.
10. Mao G, Metzger S, Lochhead MJ. "Functional Surface Coating". U.S. Patent 6,844,028; 2005
11. Harbers GM, Emoto K, Greef C, Metzger SW, Woodward HN, Mascali JJ, Grainger DW, Lochhead MJ. Functionalized poly(ethylene glycol)-based bioassay surface chemistry that facilitates bio-

immobilization and inhibits nonspecific protein, bacterial, and mammalian cell adhesion. *Chem Mater* 2007; 19:4405-4414.

12. Busscher HJ, Van der Mei HC. Microbial adhesion in flow displacement systems. *Clin Microbiol Rev* 2006; 19:127–141.

13. Roosjen A, Boks NP, Van der Mei HC, Busscher HJ, Norde W. Influence of shear on microbial adhesion to PEO brushes and glass by convective-diffusion and sedimentation in a parallel plate flow chamber. *Coll Surf B: Biointerfaces* 2005; 46:1-6.

14. Harris LG, Richards RG. Staphylococci and implant surfaces: a review. *Injury* 2006;37(2-sp1):S3-S14

15. Gottenbos B, Busscher HJ, Van der Mei HC, Nieuwenhuis P. Pathogenesis and prevention of biomaterial centered infections. *J Mater Sci Mater Med* 2002; 13:717-722.

16. Bridgett MJ, Davies MC, Denyer SP. Control of staphylococcal adhesion to polystyrene surfaces by polymer surface modification with surfactants. *Biomaterials* 1992; 13:411-416.

17. Marsh LH, Coke M, Dettmar PW, Ewen RJ, Havler M, Nevell TG, Smart JD, Smith JR, Timmins B, Tsibouklis J, Alexander C. Adsorbed poly(ethyleneoxide)-poly(propyleneoxide) copolymers on synthetic surfaces: Spectroscopy and microscopy of polymer structures and effects on adhesion of skin-borne bacteria. *J Biomed Mater Res* 2002; 61:641-652.

18. Harris LG, Tosatti S, Wieland M, Textor M, Richards RG. *Staphylococcus aureus* adhesion to titanium oxide surfaces coated with non-functionalized and peptide-functionalized poly(L-lysine)-grafted-poly(ethylene glycol) copolymers. *Biomaterials* 2004; 25:4135-4148.

19. Ista LK, Fan H, Baca O, Lopez GP. Attachment of bacteria to model solid surfaces: oligo(ethylene glycol) surfaces inhibit bacterial attachment. *FEMS Microbiol Lett.* 1996; 142:59-63.

20. Ki DP, Young SK, Dong KH, Young HK, Eun HBL, Hwal S, Kyu SC. Bacterial adhesion on PEG modified polyurethane surfaces. *Biomaterials* 1998; 19:851-859.

21. Kingshott P, Wei J, Bagge-Ravn D, Gadegaard N, Gram L. Covalent attachment of poly(ethylene glycol) to surfaces, critical for reducing bacterial adhesion. *Langmuir* 2003; 19:6912-6921.

22. Roosjen A, Busscher HJ, Norde W, Van der Mei HC. Bacterial factors influencing adhesion of *Pseudomonas aeruginosa* strains to a poly(ethylene oxide) brush. *Microbiology* 2006; 152:2673-2682.

23. Nomura S, Lundberg F, Stollenwerk M, Nakamura K, Ljungh A. Adhesion of staphylococci to polymers with and without immobilized heparin in cerebrospinal fluid. *J Biomed Mater Res* 1997; 38:35–42.

24. Van Nieuw Amerongen A. Speeksel: vorming en secretie. In: Van Nieuw Amerongen A, editor. Speeksel en mondgezondheid. Amersfoort: VU uitgeverij; 1994. p 7–37.

25. Tegoulia VA, Cooper SL. *Staphylococcus aureus* adhesion to self-assembled monolayers: effect of surface chemistry and fibrinogen presence. *Coll Surf B: Biointerfaces* 2002; 24:217–228.

Chapter 4

In Vitro and *In Vivo* Comparisons of Staphylococcal Biofilm Formation on a Crosslinked Poly(ethylene glycol)-based Polymer Coating

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Abstract

Poly(ethylene-glycol) (PEG) coatings are known to reduce microbial adhesion in terms of numbers and binding strength. However, bacterial adhesion remains in the order of 10^4 cm^{-2}. It is unknown whether these numbers of bacteria will eventually grow into a biofilm. This study investigates the kinetics of staphylococcal biofilm formation on a commercially produced, robust, crosslinked PEG-based polymer coating (OptiChem®) *in vitro* and *in vivo*. OptiChem® inhibits biofilm formation *in vitro*, and although adsorption of plasma proteins encourages biofilm formation, microbial growth kinetics are still strongly delayed compared to uncoated glass. *In vivo*, OptiChem®-coated and bare silicone rubber samples were inserted into an infected murine subcutaneous pocket model. In contrast to bare silicone rubber, OptiChem® samples did not become colonized upon re-implantation despite the fact that surrounding tissues were always culture-positive. We conclude that the commercial OptiChem® coating considerably slows down bacterial biofilm formation both *in vitro* and *in vivo*, making it an attractive candidate for biomaterials implant coating.

Introduction

Despite ongoing synthetic and engineering innovation, biomaterial surfaces have remained prone to microbial colonization during and after implantation. Biomaterial-associated infection (BAI) is a clinically significant problem affecting the success of biomaterial implants in many tissues sites, and simultaneously represents a serious health threat.[1-3] BAI is initiated by the adhesion and subsequent growth of microorganisms to an implant surface. Organisms adherent on implant surfaces can grow to form biofilms in which they are encased in a hydrated matrix of extracellular polymeric substances.[4] In essence, the biofilm mode of growth constitutes a survival mechanism for microorganisms by allowing adhering pathogens to evade the host immune system, and by finding protection within hostile milieus, even in the presence of antibiotics.[5,6] These types of medical biofilms represent a substantial challenge for successful treatment and often require implant device removal followed by systemic antimicrobial therapies to clear infections at substantial cost and morbidity. Most often, infection persists until the implant is removed, while the prospects of a revision surgery are lower than those of any primary implant because the surrounding tissue may remain compromised by bacterial presence.[7] Thus, BAI causes an enormous burden on the patient and the treating physician, with high associated costs to the health-care system. In an effort to reduce the incidence of BAI, a vast number of anti-adhesive and/or antimicrobial coatings continue to be reported to minimize microbial adhesion and subsequent biofilm formation on biomaterials surfaces. Polyethylene glycol (PEG) coatings have been extensively studied for their anti-adhesive properties,[8,9] and especially when in a brush-like configuration are known to produce significant reductions (i.e. by several orders of magnitude) in the adhesion of a wide variety of different microbial strains and species, both in terms of adhesion numbers as well in terms of adhesion strength.[9,10] Microbial adhesion from buffered solutions and also after prolonged exposure of these coatings to saliva, urine or human plasma, is shown to depend primarily on the density of the polymer brush and the length of the polymer chains determining the ability of the brush to withstand protein adsorption.[9] Such anti-adhesive properties have been attributed to their high hydration capacity that enables a

steric barrier effective in preventing microorganisms and proteins from approaching the surface. Thus, microbial adhesion is reduced by repulsive osmotic interactions that weaken binding forces and is thermodynamically unfavorable. Because of these attributes, polymer brush-coatings have been advocated as a promising approach to reduce BAI.[9,11]

Although polymer brush-coatings are generally considered non-adhesive to microorganisms, in practice this means approximately 10^4 bacteria per cm^2 remain adherent to such surfaces which may or may not be sufficient to prevent biofilm formation. Since the fate of these organisms largely depends on whether environmental conditions favor their growth on the surface, understanding adherent bacterial phenotypes and behaviors on surfaces is critical to their threats to BAI. Interestingly, to date, little is known about the resistance of PEG-based coatings to microbial growth, critical numbers of bacteria required to promote clinical infection, and subsequent biofilm formation, all factors of great importance to establish their utility in different clinical applications.

OptiChem® (Accelr8 Technology Corporation, Denver, USA) is a robust, multi-component, crosslinked PEG-based polymer surface coating that has a polymer brush configuration limited in the coating by network formation and crosslinking. It is commercially applied to solid substrates in a single-step solvent-casting process.[12-14] Compared to the more common grafted PEGs, PEG brushes, and self-assembled monolayers, this polymer film is chemically and physically thicker and more robust which, in the context of medical implants, would allow more convenient, rigorous surgical handling. An additional feature of this polymer film is that the surface chemistry can be modified *in situ* to provide diverse but specific functional coupling chemistries, lithographic patterning, and bio-immobilization capabilities within the same low non-specific binding coating matrix, useful for commercial microarray diagnostics, biosensors and selective cell adhesion studies.[12-14] In a previous study, we have shown *in vitro* that OptiChem® is effective in reducing the adhesion of different clinical bacterial isolates by several orders of magnitude in different biological media such as saliva, urine, plasma.[10] The objective of this current work is to determine to what extent OptiChem® coatings resist *in vitro* and *in vivo* biofilm formation by *Staphylococcus*

epidermidis and *Staphylococcus aureus*, two of the most common pathogens involved in BAI.

Materials and methods

Substrata

Commercially available inert OptiChem®-coated glass slides and OptiChem®-coated medical grade silicone rubber discs (diameter 8 mm; 0.5 mm thick) were used in this study. Both surface and bulk chemistry and coating properties of this PEG-based polymer coating on different substrates (e.g. modified borosilicate glass, tissue culture polystyrene, indium tin oxide, and SiO_2/Si wafers) have been previously reported in detail.[12,13] Briefly, OptiChem® (Accelr8 Technology Corporation, Denver, USA) is a poly(ethylene glycol)-based coating comprising three soluble base components that react upon casting and curing to form a stable and resilient functional crosslinked coating network with substrate adhesion and covalent attachment mechanisms. After application, the coating results in a thin, transparent film on the substratum. OptiChem® was applied on the glass slides by spin-coating whereas silicone rubber discs were plasma treated and then spin-coated. The substrata were cured and stored at -20ºC. Before implantation, all samples were sterilized in 70% ethanol, washed with sterile demineralized water and kept overnight in sterile demineralized water.

For *in vitro* biofilms, bare glass was used as a control, because its transparency allows easy use in the flow chamber described below. For *in vivo* studies, medical grade silicone rubber discs (diameter 8 mm; 0.5 mm thick) were prepared by Medin (Groningen, The Netherlands) and used as a control. Both glass and silicone rubber surfaces were cleaned to remove any surface contamination in 2% RBS 35 (Omnilabo International BV, Breda, The Netherlands) detergent solution under sonication, thoroughly rinsed with water, cleaned with methanol and washed with demineralized water again. All samples were sterilized with 70% ethanol and rinsed with sterile demineralized water. Bacterial growth was assessed prior to and after exposure of the samples to pooled citrated human blood plasma for 24 h at 37°C.

Bacterial strains and growth conditions

The clinically isolated *Staphylococcus epidermidis* 3399, and *Staphylococcus aureus* Xen29 (ATCC12600) were used *in vitro* and *in vivo*, respectively, since both strains have been often associated as the leading cause of BAI.[5,7,15] Furthermore, *S. epidermidis* has shown a relatively high affinity for inert OptiChem® coatings compared to other clinical isolates.[10] The strains were first grown from a frozen stock on blood agar plates incubated for 24 h at 37°C in ambient air and subsequently kept at 4°C. For *S. epidermidis*, one colony was used to inoculate 10 ml of Tryptone Soya Broth (TSB, OXOID, Basingstoke, England) incubated for 24 h at 37°C and used to inoculate a second culture in 200ml TSB. After overnight growth (16 h) bacteria were harvested by centrifugation (5 min at 5000 g at 10°C) and washed twice with demineralized water. Bacteria were sonicated intermittently on ice (20 s) to break bacterial chains and aggregates and obtain single cells. Bacteria were resuspended in 200 ml sterile phosphate buffered saline (PBS, 10mM potassium phosphate, 150mM NaCl, pH 6.8), supplemented with 2% TSB, pH 7. For *S. aureus*, one colony was used to inoculate 10 ml TSB and incubated overnight under the same conditions as described above. This culture (100 µL) was used to inoculate a second culture containing 10 ml of TSB supplemented with 4% NaCl, in order to stimulate biofilm growth. Thirteen bare silicone rubber discs were subsequently incubated for a period of 72 h at 37°C under stirring at 60 rpm to grow biofilms on these discs. These discs with a biofilm were implanted into mice to induce BAI.

In vitro *kinetics of biofilm formation*

Bacterial adhesion and growth on samples with and without adsorbed plasma proteins under laminar flow were assessed using real-time (*in situ*) image analysis in a parallel plate flow chamber (175 mm length x 17 mm width x 0.75 mm depth).[16] Before each experiment, the entire system was filled with sterile buffer (PBS, supplemented with 2% TSB) to remove all air-bubbles from the tubes and flow chamber. Flow rates were adjusted to 0.48 ml s^{-1} (corresponding with a wall shear rate of 5 s^{-1}) for 60 min while the flow chamber was warmed up to 37°C. The flow rate was kept constant throughout an entire experiment. Then, buffer flow was switched to a bacterial suspension that

circulated for 90 min in order to allow bacteria to adhere to the substratum. Because adhesion of *S. epidermidis* 3399 to OptiChem®-coated slides is very low compared to glass,[10] the bacterial concentration in suspension was set to 12 x 10^8 per ml for experiments on OptiChem®-coated slides, and to 0.2 x 10^8 per ml for experiments on glass, in order to reach a similar surface density before biofilm growth starts on both glass and OptiChem® coatings within the same time span. After perfusion with bacterial suspension for 90 min, the flow was switched once more to sterile buffer to remove unbound bacteria from the tubes and chamber during 30 min, and ultimately switched to 100% TSB to induce overnight growth (16 h).

Bacterial adhesion and subsequent growth were studied on the bottom plate of the flow chamber, consisting of the material under study (i.e. glass or OptiChem®-coated glass slides, either with or without adsorbed plasma proteins). Adhesion and growth were followed with a CCD-MXRi camera (Hight Technology, Eindhoven, The Netherlands) mounted on a phase contrast microscope (Olympus BH-2). Images of the bottom plate were collected from t = 0, (i.e., the moment the flow chamber was initially perfused with full medium), at 20 min time intervals. In order to distinguish between adhesion, growth of adhering bacteria and detachment, bacteria present on the substrata at each point of time were registered on a template for tracking and comparison against bacterial presence in subsequent images. Experiments were carried out three times with separately grown bacteria cultures on each substratum.

Biofilm morphology and viability

Confocal Laser Scanning Microscopy (CLSM), (Leica TCS SP2, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) was used to determine the morphology and viability of biofilms formed on the different surfaces. Biofilms were stained with a live/dead stain (Live/Dead Baclight bacterial viability kit; Molecular Probes, Leiden, The Netherlands), followed by an incubation period of 30 min at room temperature in the dark. Biofilms were immersed in buffer and observed using a 40x water immersion objective. The lasers were set for tetramethylrhodamine isocyanate (excitation at 543 nm and emission at 560 to 700 nm) and fluorescein isothiocyanate (excitation at 488 nm and emission at 500 to 600 nm).

In vivo *murine implant model*

Biofilm formation on inert OptiChem®-coated and bare silicone rubber, a common implant material, were compared in a murine revision surgery model. In this model, BAI was first induced and attempted to be treated with antibiotics, followed by the insertion of a new, sterile implant. Silicone rubber was chosen because it is an often-used clinical biomaterial, for example in reconstructive surgery, voice prostheses, breast implants, and vascular and urinary catheters.[17] Furthermore, it has often been associated with high bacterial colonization rates.[18,19]

Colonized silicone rubber discs were implanted subcutaneously in pockets made in the left flank of thirteen female Balb/c OlaHsd mice (6-8 weeks old, Harlan Netherlands BV, Horst, The Netherlands), as described by Engelsman *et al*.[20] During induction of BAI, mice were treated daily with intraperitoneal injections (0.5 ml) of vancomycin (2 mg ml^{-1}, Abbot BV, Hoofdorp, The Netherlands) and rifampicin (1 mg ml^{-1}, Rifadin, Aventis, Hoevelaken, The Netherlands). After 4 days, antibiotic treatment was stopped and the colonized discs were surgically replaced by either a sterile silicone rubber or an OptiChem®-coated disc, modeling an implant revision surgery. Animals were sacrificed after another 5 days, discs were removed and a biopsy from the tissue (including a portion of muscle) surrounding the discs was taken. All discs and biopsies were collected in reduced transported fluid (per liter: NaCl (0.9 g) $(NH_4)_2SO_4$ (0.9 g), KH_2PO_4 (0.45 g), Mg_2SO_4 (0.19g), K_2HPO_4 (0.45 g), Na_2EDTA (0.37 g), L-cysteine HCl (0.2 g), pH 6.8). All animal experiments were approved by the Animal Experiment Committee, University of Groningen, The Netherlands.

Ex vivo *implant analyses*

All discs and tissue samples were sonicated three times (10 s) to detach staphylococci from the discs or obtain a tissue homogenate. The numbers of colony forming units (CFU) on the explanted discs or in the tissue homogenates were determined by plating 100 μL of the bacterial suspension or homogenate, respectively, on blood agar plates and incubating for 24 h at 37°C. Tissue homogenate was tested for the presence of antibiotic levels. To this end, 20 μL of homogenate were placed on Mueller-Hinton agar plates inoculated with *S. aureus* Xen29. Growth inhibition was determined visually after

incubation for 24 h at 37°C. No detectable effective antibiotic levels remained in any of the excised tissue samples.

Statistics analysis

Statistically significant differences ($p < 0.05$) between biofilms on OptiChem®-coated glass and bare glass were determined between the means of the two groups by the two-tailed Student's t-test.

Results

In vitro *kinetics of biofilm formation*

Flow chamber results *in vitro* showed that *S. epidermidis* 3399 adhered from buffer to the different substrata, yielding a surface density of approximately 0.3×10^6 cm^{-2} after 90 min. After introducing growth medium into the flow chamber (t = 0), the kinetics of staphylococcal biofilm formation were monitored for 240 min, carefully distinguishing adhesion of newly arriving bacteria, (i.e. bacteria that had detached up-stream and re-adhered downstream), growth of adhering bacteria and detachment, as shown in Figure 1, by use of digital image processing. On glass, both bacterial adhesion and growth took place at high rates and led to a rapid increase in the total number of resident staphylococci, and the formation of biofilms characterized by more mature, adherent colonies. On OptiChem®, bacterial adhesion and growth were significantly reduced, while further detachment was high, yielding a low total number of adhering bacteria after 240 min in growth media. Interestingly, adsorption of plasma proteins enhanced adhesion and growth on OptiChem® while on glass adhesion and growth were suppressed. Yet, ultimate biofilm growth on plasma-coated OptiChem® was significantly delayed compared to glass due to higher staphylococcal detachment rates from plasma-coated OptiChem®. Growth medium was further allowed to circulate through the flow chamber up to 960 min in order to study the morphology and viability of the more mature biofilms. Figure 2 shows confocal laser scanning microscopy (CLSM) images of the different biofilms after 960 min of growth in the flow chamber, and reports the

viability of *S. epidermidis* 3399 on each surface by virtue of the fluorescence colors. On glass, dense biofilms were observed with 99 ± 1% of the staphylococci alive. In contrast, biofilms on OptiChem® consisted of scattered microcolonies, where viability had decreased to 73 ± 14%. Adsorption of blood plasma proteins on OptiChem® led to biofilms after 960 min, but biofilms were still less dense and slightly less viable (88 ± 7 %) than biofilms found on plasma-coated glass (92 ±7 %).

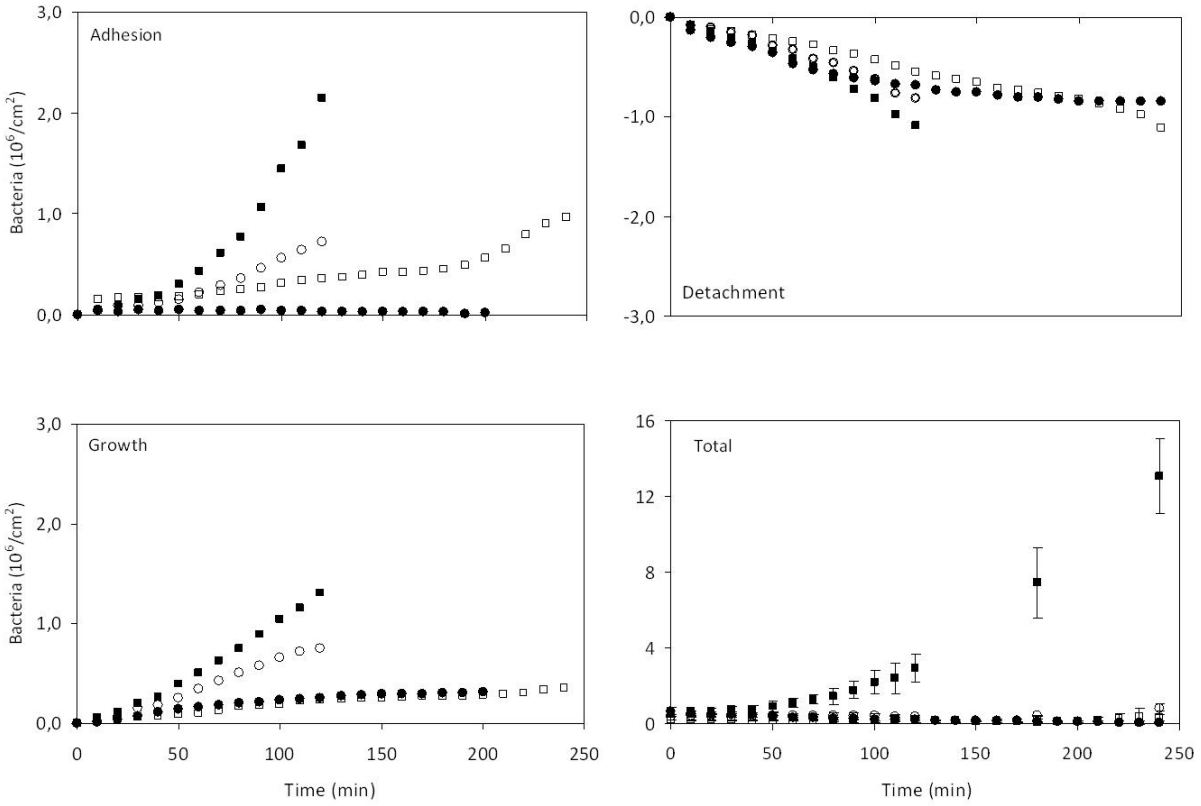

Figure 1. Numbers of *S. epidermidis* 3399 adherent on surfaces as a function of time after introducing growth medium into the flow chamber: (■) bare glass, (●) bare OptiChem®, (□) plasma-coated glass, (○) plasma-coated OptiChem®. Note that adhesion, growth and detachment are simultaneous processes individually reported here. Error bars represent standard deviations of three measurements.

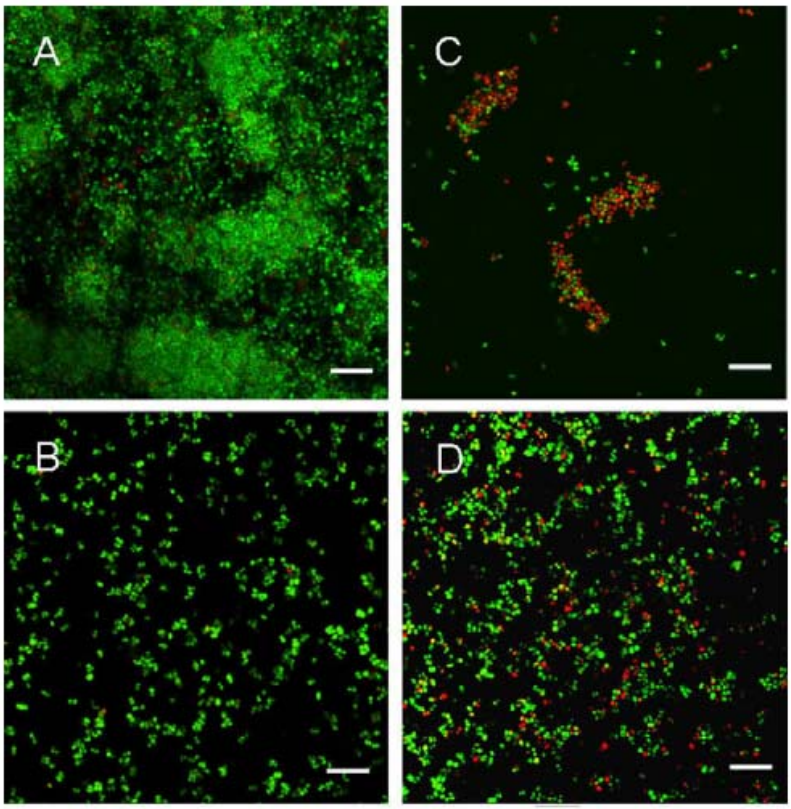

Figure 2. Selected CLSM images of *S. epidermidis* 3399 biofilms after 960 min of flow in growth media and staining with live-dead fluorescent dyes on glass (left panel) and OptiChem®-coated glass (right panel) without (top, A,C) and with (bottom, B,D) an adsorbed film of plasma proteins. Green and red dots represent live and dead bacteria, respectively. Scale bar corresponds to 10 µm.

In vivo *bacterial adhesion and biofilm formation on OptiChem®*

Bacterial adhesion and biofilm formation on and around implanted OptiChem®-coated silicone rubber discs was compared with adhesion and biofilm formation on and around bare silicone rubber discs in a murine infected subcutaneous implant pocket model. Figure 3 shows that adhesion of *S. aureus* to OptiChem®-coated discs was not detectable (0 out of 7) in contrast to bare silicone rubber discs that appeared nearly all colonized (5 out of 6). Surrounding tissue was culture–positive in all cases, except for one OptiChem®-coated disc, with no significant differences in the numbers of colony forming units (CFUs) counted between both groups.

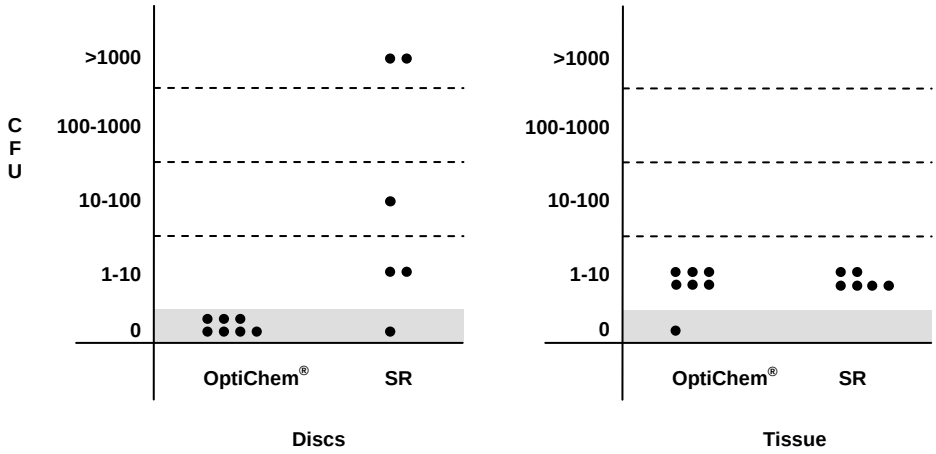

Figure 3. Frequencies of culture positive samples and numbers of *S. aureus* CFUs present on explanted OptiChem®-coated and bare silicone rubber discs (left) and in surrounding tissues biopsies (right) after revision surgery. Shaded area represents the detection limit.

Discussion

The utility of PEG coatings to reduce bacterial adhesion is well-recognized and consistently practiced and reported.[8,9,21,22,24] However, there is a distinct difference between bacterial adhesion *in vitro* and biofilm formation leading to implant-related infection *in vivo*. This critical distinction is not often investigated and *in vitro* studies fail to correlate with or recognize the importance of *in vivo* results. This perhaps is a primary hindrance to the field with respect to understanding infection of biomaterials. In this paper, we have investigated the kinetics of bacterial biofilm formation on a commercially available crosslinked PEG-based polymer coating (OptiChem®) using *in vitro* and *in vivo* models. Interestingly, no correlation between biofilms formed *in vitro* and *in vivo* was found. An OptiChem® coating effectively inhibited biofilm formation *in vitro* during 960 min of growth in a well-characterized flow chamber, while the adsorption of plasma proteins produced a small loss of the anti-adhesive coating activity. Biofilms produced *in vitro* were slightly less viable on the coating than on glass as shown by a fluorescent live/dead assay. *In vivo*, OptiChem®-coated silicone rubber discs implanted in murine infected subcutaneous pockets did not become colonized by staphylococci, while bare silicone rubber discs were consistently colonized.

Bacterial adhesion was reduced on OptiChem® *in vitro* as shown previously,[10] and this was coupled to a strong delay in biofilm formation, as well as to a strong infection resistance *in vivo*. OptiChem® has a sub-optimally organized PEG brush surface configuration, and together with its high hydration capacity, yields weak interfacial interaction forces with bacteria, producing low adhesion numbers and high detachment rates and thus reduced biofilm formation. Currently, only a few studies have reported biofilm formation on PEG brushes *in vitro*.[21,22] Cheng *et al.*[22] showed that adhesion and biofilm formation by *S. epidermidis* and *Pseudomonas aeruginosa* was reduced on poly(oligo(ethylene glycol) methyl ether methacrylate) brushes, but their assessments may have been influenced by removal of substrate samples through the air-aqueous interface, thereby causing detachment of adhering bacteria by the substantial surface tension forces at the liquid-air interface. Generally, these surface tension forces are higher than forces governing bacterial adhesion to polymer brushes.[25,26] Biofilm formation by several bacterial strains and their viability on surfaces comprising adsorbed tri-block copolymers of polyethylene oxide (PEO) and polypropylene oxide (PPO) brushes on silicone rubber were recently reported by Nejadnik *et al*.[21] Their biofilms developed slowly *in vitro* on tri-block copolymer brushes compared to those on pristine silicone rubber, a result entirely consistent with results in this paper, although biofilms were more viable on the tri-block copolymer brush than on bare silicone rubber. The higher microbial viability on the tri-block copolymer brushes was attributed to more ready diffusion of nutrients from the media into microcolonies compared to denser biofilms found on pristine silicone rubber. Biofilms on OptiChem®-coated glass slides were also highly viable, similar to that observed for biofilms on the tri-block copolymer brushes on silicone rubber. In contrast, biofilms on hydrophilic glass in the absence of a polymer brush coating were highly viable compared to biofilms on the hydrophobic silicone rubber (exhibiting less than 50% viable bacteria).[21]

Medical devices implanted into the body instantly adsorb a complex heterogeneous protein layer from the surrounding tissue onto the implant surface. We have simulated this in the *in vitro* experiments by exposing OptiChem® to blood plasma proteins before starting bacterial adhesion. Proteins pre-adsorbed on the coating enhanced bacterial adhesion, growth and detachment rates with the net effect of reducing the non-adhesive

functionality of the brush coating.[10] On glass, the effect was the opposite. In general, PEG-based coatings are known to significantly reduce protein adsorption, but there is some evidence that particularly small proteins from blood plasma or serum can penetrate into PEG brushes, and remain there,[23,24] affecting bacterial adhesion.[9,10,24,27,28] Furthermore, Tedjo *et al*.[24] suggested that proteins adsorbed onto PEG brushes undergo conformational changes, allowing cells and bacteria to attach to the surface. In addition, formation of bacterial aggregates in the presence of plasma proteins was observed on the OptiChem® surface during bacterial growth, possibly resulting from fibrinogen-recognizing adhesins present on the staphylococcal cell wall interacting with adsorbed fibrinogen molecules.[24,28,29]

Infection recurrence after implant revision surgery is a common clinical problem.[30,31] Broekhuizen *et al*.[7] showed that tissue adjacent to colonized implants in mice was compromised, and that tissue infection persisted after treatment with systemic rifampicin/vancomycin. Accordingly, tissues became a focus as a reservoir of bacteria that re-seed surgical sites and re-colonize implants after revision surgery. The *in vivo* model used in this study closely mimicked the clinical procedural treatment of a BAI with antibiotics followed in revision surgery, mandating implant removal and replacement. In agreement with current literature, we also observed that tissues adjacent to the implanted discs were always culture-positive, regardless of whether the discs were OptiChem®-coated or not. Furthermore, biofilms were always harvested from pristine silicone rubber discs, consistent with clinical studies reporting high colonization rates of silicone rubber and recurrence of infection after revision surgery.[18,21,31] OptiChem®-coated discs, on the other hand, remained effective against bacterial adhesion upon re-implantation, and no bacteria were harvested from coated discs, demonstrating the efficacy of the coating to resist biofilm formation. Clinically, this is of great importance, as the biomaterial is generally considered one source of microorganisms from which adjacent tissue becomes infected, or vice-versa.[7]

Apart from stimulating BAI, another feature that limits the use of biomaterial coatings is the lack of tissue integration.[32] Successful tissue integration of biomaterials is defined by many as “a race for surface” since proteins, bacteria and host cells all compete for colonization of the implant surface niche.[32-34] This highlights the need for bi-functional

surfaces that promote tissue integration while at the same time inhibiting non-specific microbial adhesion. Tissue integration can be encouraged on biomaterial surfaces by attaching chemistry and immobilized proteins or peptides selective toward promoting adhesion of a unique or multiple host cell types, according to the final application. Also OptiChem® coatings can be modified to provide the ability of selective bio-immobilization of desired molecules within the same low non-specific binding coating matrix,[12] for example, with cell integrin-specific arginine-glycine-aspartic acid (RGD), a short peptide sequence common to cell matrix proteins such as fibronectin and vitronectin, and recognized by integrin receptors located on focal adhesion sites on the cell membrane.[35]

Conclusion

As a commercial, relatively thick, chemically stable and robust coating, OptiChem® has proven utility as a PEG-based biomaterial coating for mitigation of BAI, limiting initial growth and preventing recurrence of infection after revision surgery.

References

1. Rohde H, Mack D, Christner M, Burdelski C, Franke G, Knobloch J. Pathogenesis of staphylococcal device-related infections: from basic science to new diagnostic, therapeutic and prophylactic approaches. *Rev Med Microbio* 2006; 17:45-54.

2. Göyz F, Peters G. Colonization of medical devices by coagulase-negative staphylococci. In: Waldvogel FA, Bisno AL, editors. Infections associated with indwelling medical devices, 3rd ed. Washington DC: ASM Press; 2000. p 55-88.

3. Anderson JM, Marchant RE. Biomaterials: factors favoring colonization and infection. In: Waldvogel FA, Bisno AL, editors. Infections associated with indwelling medical devices, 3rd ed. Washington DC: ASM Press; 2000. p 89- 110

4. Sauer K, Rickard AH, Davies DG. Biofilms and biocomplexity. *Microbe* 2007; 2:347-353.

5. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbio* 2004; 2:95-108.

6. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002; 15:167-193.

7. Broekhuizen CA, de Boer L, Schipper K, Jones CD, Quadir S, Feldman RG, Dankert J, Vandenbroucke-Grauls CM, Weening JJ, Zaat SA. Peri-implant tissue is an important niche for *Staphylococcus epidermidis* in experimental biomaterial-associated infection in mice. *Infect Immun* 2007; 75:1129-1136.

8. Park KD, Kim YS, Han DK, Kim YH, Lee EH, Suh H, Choi KS. Bacterial adhesion on PEG modified polyurethane surfaces. *Biomaterials* 1998; 19:851-859.

9. Roosjen A, De Vries J, Van der Mei HC, Norde W, Busscher HJ. Stability and effectiveness against bacterial adhesion of PEO coatings in biological fluids. *J Biomed Mater Res B App .Biomater* 2005; 73:347-354.

10. Saldarriaga Fernández IC, Van der Mei HC, Lochhead MJ, Grainger DW, Busscher HJ. The inhibition of the adhesion of clinically isolated bacterial strains on multi-component cross-linked poly(ethylene glycol)-based polymer coatings. *Biomaterials* 2007; 28:4105-4112.

11. Currie EPK, Norde W, Stuart MAC. Tethered polymer chains: surface chemistry and their impact on colloidal and surface properties. *Adv Coll Interf Sc* 2003; 100-102:205-265.

12. Harbers GM, Emoto K, Greef C, Metzger SW, Woodward HN, Mascali JJ, Grainger DW, Lochhead MJ. Functionalized poly(ethylene glycol)-based bioassay surface chemistry that facilitates bio-immobilization and inhibits nonspecific protein, bacterial, and mammalian cell adhesion. *Chem Mater* 2007; 19:4405-4414.

13. Takahashi H, Emoto K, Dubey M, Castner DG, Grainger DW. Imaging surface immobilization chemistry: correlation with cell patterning on non-adhesive hydrogel thin films. *Adv Funct Mater* 2008; 18:2079-2088.

14. Mao G, Metzger S, Lochhead MJ. US patent 6,844,028, 2005

15. Shive MS, Hasan SM, Anderson JM. Shear stress effects on bacterial adhesion, leukocyte adhesion, and leukocyte oxidative-11e capacity on a polyetherurethane. *J Biomed Mater Res* 1999; 46:511-519.

16. Busscher HJ, Van der Mei HC. Microbial adhesion in flow displacement systems. *Clin Microbiol Rev* 2006; 19:127-141.

17. Curtis J, Colas A. Medical applications of silicone. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, editors. Biomaterials Science, 2nd ed. San Diego, CA: Elservier Academic Press; 2004. p 697-707.

18. Darouiche RO. Device-associated infections: A macroproblem that starts with microadherence. *Clin Infect Dis* 2001; 33:1567-72.

19. Eerenstein SEJ, Grolman W, Schouwenburg PF. Microbial colonization of silicone voice prostheses used in laryngectomized patients. *Clin Otolaryngol Allied Sci* 1999; 24:398-403.

20. Engelsman AF, Van der Mei HC, Francis KP, Busscher HJ, Ploeg RJ, van Dam GM. Real time noninvasive monitoring of contaminating bacteria in a soft tissue implant infection model. *J Biomed Mater Res B Appl Biomater* 2009; 88:123-129.

21. Nejadnik MR, Van der Mei HC, Norde W, Busscher HJ. Bacterial adhesion and growth on a polymer brush-coating. *Biomaterials* 2008; 29:4117-4121.

22. Cheng G, Zhang Z, Chen S, Bryers JD, Jiang S. Inhibition of bacterial adhesion and biofilm formation on zwitterionic surfaces. *Biomaterials* 2007; 28:4192-4199.

23. Zhang M, Desai T, Ferrari M. Proteins and cells on PEG immobilized silicon surfaces. *Biomaterials* 1998; 19:953-960.

24. Tedjo C, Neoh KG, Kang ET, Fang N, Chan VJ. Bacteria-surface interaction in the presence of proteins and surface attached poly(ethylene glycol) methacrylate chains. *J Biomed Mater Res A* 2007; 82:479-491.

25. Gomez-Suarez C, Busscher HJ, Van der Mei HC. Analysis of bacterial detachment from substratum surfaces by the passage of air-liquid interfaces. *Appl Environ Microbiol* 2001; 67:2531-2537.

26. Roosjen A, Busscher HJ, Norde W, Van der Mei HC. Bacterial factors influencing adhesion of *Pseudomonas aeruginosa* strains to a poly(ethylene oxide) brush. *Microbiology* 2006; 152:2673-2682.

27. Efremova NV, Sheth SR, Leckband DE. Protein-induced changes in poly(ethylene glycol) brushes: molecular weight and temperature dependence. *Langmuir* 2001; 17:7628-7636.

28. Patel JD, Ebert M, Ward R, Anderson JM. *S. epidermidis* biofilm formation: effects of biomaterial surface chemistry and serum proteins *J Biomed Mater Res A* 2007; 80:742-751.

29. Nilsson M, Frykberg L, Flock JI, Pei L, Lindberg M, Guss B. A fibrinogen-binding protein of *Staphylococcus epidermidis. Infect Immun* 1998; 66:2666-2673.

30. Went P, Krismer M, Frischhut B. Recurrence of infection after revision of infected hip arthroplasties. *J Bone Joint Surg Br* 1995; 77-B:307-309.

31. Darouiche RO. Current concepts: treatment of infections associated with surgical implants. *N Engl J Med* 2004; 350:1422-1429.

32. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* 1987; 237:1588-1595.

33. Schierholz JM, Beuth J. Implant infections: a haven for opportunistic bacteria. *J Hosp Infect* 2001;49:87-93.

34. Schmidt AH, Swiontkowski MF. Pathophysiology of infections after internal fixation of fractures. *J Am Acad Orthop Surg* 2000; 8:285-291.

35. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. *Science* 1987; 238:491-497.

Chapter 5

Simultaneous Bacterial and Tissue Cell Interactions on Crosslinked Poly(ethylene glycol)-based Polymer Coatings

Submitted to JBMR part A. Isabel C. Saldarriaga Fernández, Henk J. Busscher, Steve Metzger, David W. Grainger, Henny C. van der Mei.

Abstract

Biomaterial-associated infections (BAI) remain a serious clinical complication, often arising from an inability of host tissue-implant integration to out-compete bacterial adhesion and growth. A commercial polymer coating based on polyethylene glycol (PEG), available in both chemically unreactive and NHS-activated forms (OptiChem®), was compared for simultaneous growth of staphylococci bacteria and osteoblasts. In the absence of staphylococci, osteoblasts adhered and proliferated well on glass controls and on the NHS-reactive PEG-based coating over 48 h, but not on the unreactive PEG coating. Staphylococcal growth was low on both PEG-based coatings. When staphylococci were pre-adhered on surfaces for 1.5 h to mimic peri-operative contamination, osteoblast growth and spreading was reduced on glass but virtually absent on both reactive and unreactive PEG-based coatings. Thus although NHS-reactive, PEG-based coatings stimulated tissue-cell interactions in the absence of contaminating staphylococci, the presence of adhering staphylococci eliminated osteoblast adhesion advantages on the PEG surface. This study demonstrates the importance of using bacterial and cellular co-cultures compared to monocultures when assessing functionalized biomaterials coatings for infectious potential.

Introduction

Biomaterial-associated infections (BAI) remain a serious complication in modern medicine with devastating clinical consequences ranging from complete implant failure to lethal sepsis of the patient.[1] Economic consequences of BAI are also noteworthy, as the magnitude of BAI and requirements for resulting treatments are costly and significant.[1] As the use of implanted biomaterials continues to arise, BAI incidence, costs and morbidity will also increase. For this reason, the design of improved biomaterials or functional coatings capable of withstanding biofilm formation while simultaneously providing a strong interface with surrounding host tissue cells is essential to ensure the long-term success of many implanted biomedical devices.

Despite careful sterile and hygienic surgical suite conditions during implantation of medical devices, viable pathogens from ubiquitous human skin flora, such as *Staphylococcus epidermidis,* can enter the surgical site and contaminate the implanted device. Alternatively, airborne microorganisms may contaminate device surfaces prior to implantation.[2,3] Consequently, after device placement, host cells and microorganisms will simultaneously compete for colonization of the biomaterial surface.[4] BAI incidence will decrease if host cells rapidly adhere and readily proliferate on the biomaterial surface to out-compete bacteria at the implant site – a scenario previously called "the race for the surface".[4] As cell-surface adhesion motifs and strategies are often too general to select only mammalian cells over microbes, deliberate designs to increase affinity of a biomaterial surface or coatings for tissue cells is frequently accompanied by increased bacterial adhesion.

Many strategies have sought to reduce microbial adhesion and subsequent biofilm formation on implant surfaces, including use of hydrophobic coatings and application of quaternary ammonium (cationic) compounds.[5-8] However, these approaches adsorb many host proteins, providing a conditioning film for bacterial attachment. Due to their intrinsically low protein adsorption and hundred-fold reductions bacterial adhesion with respect to common biomaterials, poly(ethylene) glycol (PEG) coatings have become the "first choice" strategy for reducing bacterial adhesion.[9] Hydrated, sufficiently dynamic PEG-polymer chains (e.g., brushes) are also proposed to reduce bacterial

adhesion through the steric repulsion between the hydrated PEG chains.[10] Although polymer brushes are designed to rapidly hydrate and suppress non-specific adhesion of biomolecules to surfaces, these coatings can also be chemically modified to promote specific immobilization of tissue cells.[11-13] If resulting tissue cell-surface interactions are weak, non-specific, or insufficient to enable rapid mammalian cell attachment, bacterial adhesion and biofilm formation may remain insignificant and clinically unaffected.[11]

OptiChem® is a commercially available, crosslinked PEG-based coating with an amine-reactive (NHS active ester) chain-terminal chemical functionality in its reactive form to facilitate specific immobilization of biomolecules. The NHS-functionality is deactivated with methoxyethylamine to provide a non-reactive or 'chemically inert' PEG surface with very low, non-specific binding of biological molecules from physiological milieu.[14-16] Our previous studies have shown that deactivated inert OptiChem® coatings reduce adhesion of a variety of clinical bacterial isolates in different physiological fluids,[17] and delay formation of mature biofilms[18]. However, little is known about how bacteria interact with NHS-reactive PEG-based coatings, and the simultaneous growth of both bacteria and mammalian cells on inert and NHS-reactive OptiChem®.

Recently, a novel methodology has been forwarded to evaluate the simultaneous growth of tissue cells and bacteria in a single co-culture experiment under the presence of controlled, variable fluid shear and in different media.[19] The aim of this study was to compare the simultaneous growth of *S. epidermidis* and U2OS osteoblast co-cultures on both deactivated 'inert' and NHS-reactive crosslinked PEG-based (OptiChem®) *in vitro*.

Materials and methods

Substrata

Simultaneous bacterial and osteoblast cell growth in co-cultures were studied on OptiChem®-coated glass slides (Accelr8 Technology, USA, commercially available as Schott-Nexterion™ Slide H). The chemical formulation of the coating, its full characterization and bio-immobilization properties have been described previously.[14-16] OptiChem® was applied on glass slides by spin-coating and curing to crosslink the PEG

matrix.[14] Coated slides were stored continuously at -20°C prior to use. Half of the coated-slides were used in the NHS-reactive form, denoted here as "reactive OptiChem®". The remaining slides were deactivated by removal of the NHS surface groups ("inert OptiChem®") with methoxyethylamine.[14] Uncoated glass (Mezel-Gläser, 76 mm x 26 mm x 1 mm) was used as a control surface. Glass slides were cleaned in 2% RBS 35 detergent solution (Omnilabo International BV, Breda, The Netherlands) under sonication, rinsed with demineralized water, submerged in methanol, and washed once more with demineralized water. All samples were sterilized in 70% ethanol for 15 min and rinsed with sterile demineralized water and subsequently with sterile PBS.

Bacterial strain and growth conditions

S. epidermidis, a pathogen often associated with BAI,[1,20] was used to assess bacterial adhesion to the coatings. The clinical isolate, *S. epidermidis* 3399, was grown aerobically on blood agar plates from a frozen stock. One colony was used to inoculate 10 ml of tryptone soya broth (TSB, OXOID, Basingstoke, England) and incubated for 24 h at 37°C in ambient air. This culture was used to inoculate another culture in 200 ml TSB that was allow to grow overnight at 37°C. Bacteria were harvested by centrifugation (5 min at 5000 g at 10°C), and washed twice with sterile phosphate buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 6.8). Bacteria were sonicated intermittently on ice (20 s) to break aggregates and resuspended in 200 ml sterile PBS to a concentration of 3 x 10^6 bacteria ml^{-1} as determined with the Bürker-Türk counting chamber.

Cell culture conditions

U2OS osteosarcoma cells were grown in TCPS flasks (Greiner, Germany), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g l^{-1} D-glucose, pyruvate, 10% fetal bovine serum (FBS) and 0.2 mM ascorbic acid-2-phosphate (AA2P), denoted as "complete DMEM". Cells were always at 37°C in a humidified atmosphere of 5% CO_2 and passaged at 90-100% confluency using trypsin-EDTA. Prior to assessments, cells were harvested and resuspended to a concentration of 7.5 x 10^5 ml^{-1} in complete DMEM supplemented with 2% TSB (denoted in this study as

"optimal medium") shown to facilitate optimal growth of both the bacteria and osteoblast cells in co-culture.[19]

U2OS adhesion assay

U2OS adhesion to OptiChem® coatings in the presence or absence of co-seeded bacteria was assessed under laminar flow using real-time (*in situ*) image analysis in a parallel plate flow chamber.[19] The flow chamber was equipped with a CCD-MXRi camera (High Technology, Eindhoven, The Netherlands) mounted on a phase contrast microscope (Olympus BH-2). Assays were observed on the bottom plate of the flow chamber containing the substrata under study (i.e. glass, inert OptiChem® or reactive OptiChem®-coated glass slides).

Prior to each experiment, the flow system was carefully filled with sterile PBS to remove air bubbles present in the tubing and chamber, and PBS was allowed to flow for 30 min at a shear rate of 11 s^{-1}. Then, the bacterial suspension was perfused through the chamber for 90 min, keeping the flow rate constant at 11 s^{-1}. Subsequently, the flow was switched to sterile PBS and circulated for 30 min to remove unbound bacteria and the bacterial suspension from the tubing and chamber while the system was warmed to 37°C. The U2OS cell suspension was then introduced into the flow chamber. Once the entire volume of buffer inside the chamber was replaced by the cell suspension, flow was stopped for 90 min to allow osteoblast cells to settle and attach to the substrata. Ultimately, "optimal medium" supplemented with 2% HEPES was perfused through the system without recirculation at a shear rate of 0.14 s^{-1} for 48 h. The percentage surface area covered by adherent cells after 90 min of adhesion was determined. Images were collected throughout the assay timecourse. Experiments were performed in triplicate with separately grown bacterial cultures on each substratum.

Immunocytochemical assays

In order to determine the morphology, proliferation and spreading of U2OS cells after 48 h, the substrata were fixed with 3.7% formaldehyde in cytoskeleton stabilization buffer (CS; 0.1 M Pipes, 1mM EGTA, 4% (w/v) polyethylene glycol 8000, pH 6.9). Subsequently, the samples were incubated in 0.5% Triton X-100 for 3 min, rinsed with PBS, followed

by staining with DAPI and TRITC-phalloidin in PBS. After incubation for 30 min in the dark, samples were washed four times with PBS and examined with fluorescent microscopy (Leica DM 4000B). The percentage surface area covered by adherent cells after 48 h was determined using Scion image software.

Statistical analysis

Statistical ANOVA analysis was performed followed by a Tukey's HSD post-hoc test and a p-value < 0.05 was considered statistically significant.

Results

Images of U2OS cells seeded on glass, inert OptiChem® and reactive OptiChem® at 1.5 h, in the presence of pre-adhering staphylococci on each substratum are shown in Figure 1.

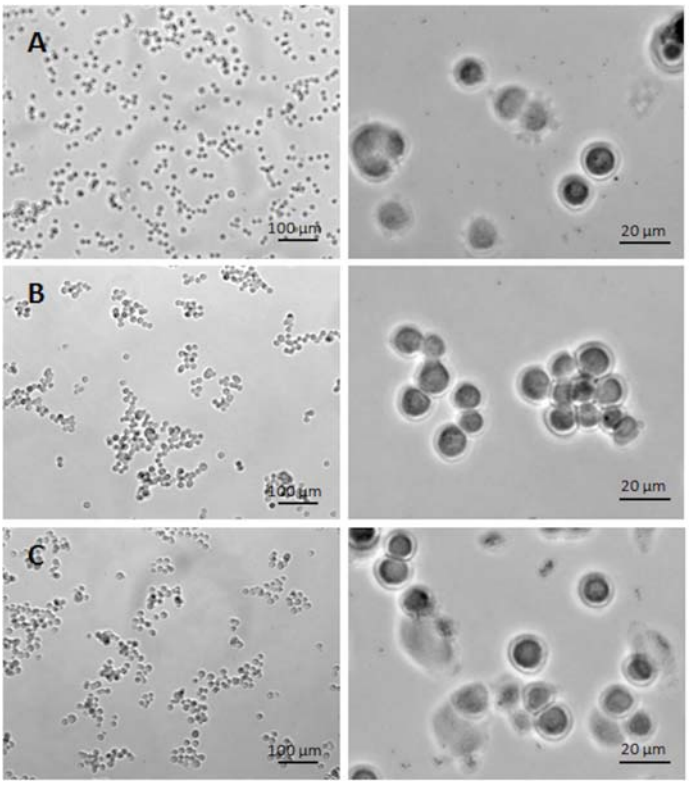

Figure 1. Phase-contrast images of bacteria and U2OS cells seeded after 1.5 h on: glass (A), inert OptiChem® (B) and reactive OptiChem® (C). The bars correspond to 100 µm (left column) and 20 µm (right column).

Mammalian cells seeded on glass were well distributed over the surface whereas cells seeded on both inert and reactive OptiChem® coatings tended to aggregate, irrespective of the presence of staphylococci. Cells seeded on glass and reactive OptiChem® attached and started spreading after 1.5 h. By contrast, cells seeded on inert OptiChem® did not adhere well; most were removed from the surface during perfusion with optimal medium, despite the low shear rate applied (0.14 s^{-1}).

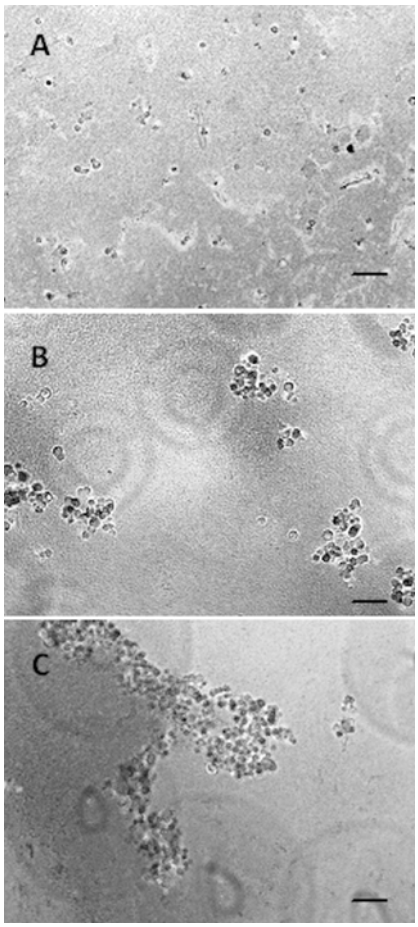

Figure 2. Phase-contrast images of U2OS cells and biofilms formed by *S. epidermidis* 3399 after 48 h on: glass (A), inert OptiChem® (B) and reactive OptiChem® (C). Dark areas are biofilms. Scale bar corresponds to 100 µm.

In the presence of pre-adherent staphylococci, a mature biofilm was observed on all substrata after 48 h of growth, as shown in Figure 2. However, biofilms formed on OptiChem® coatings were less dense and adhered weakly: these biofilms were easily removed from OptiChem® surfaces by applying a slightly higher shear rate (2 s^{-1}) at the end of the assessments. Furthermore, osteoblast cell spreading occurred on glass,

whereas significant cell spreading on both inert and reactive OptiChem® coatings was not observed.

Fluorescent microscopy images of immunostained cells after 48 h of growth on each substratum are shown. In the absence of co-cultured bacterial biofilms, osteoblast cells on glass and reactive OptiChem® spread equally well (left column). Due to the presence of *S. epidermidis*, the adhesion and spreading of U2OS cells were significantly reduced on all substrata compared to controls (i.e., monoculture controls in the absence of staphylococci). Furthermore, the few cells that managed to adhere to inert OptiChem® maintained spherical shapes and did not spread, irrespective of the absence or presence of staphylococci.

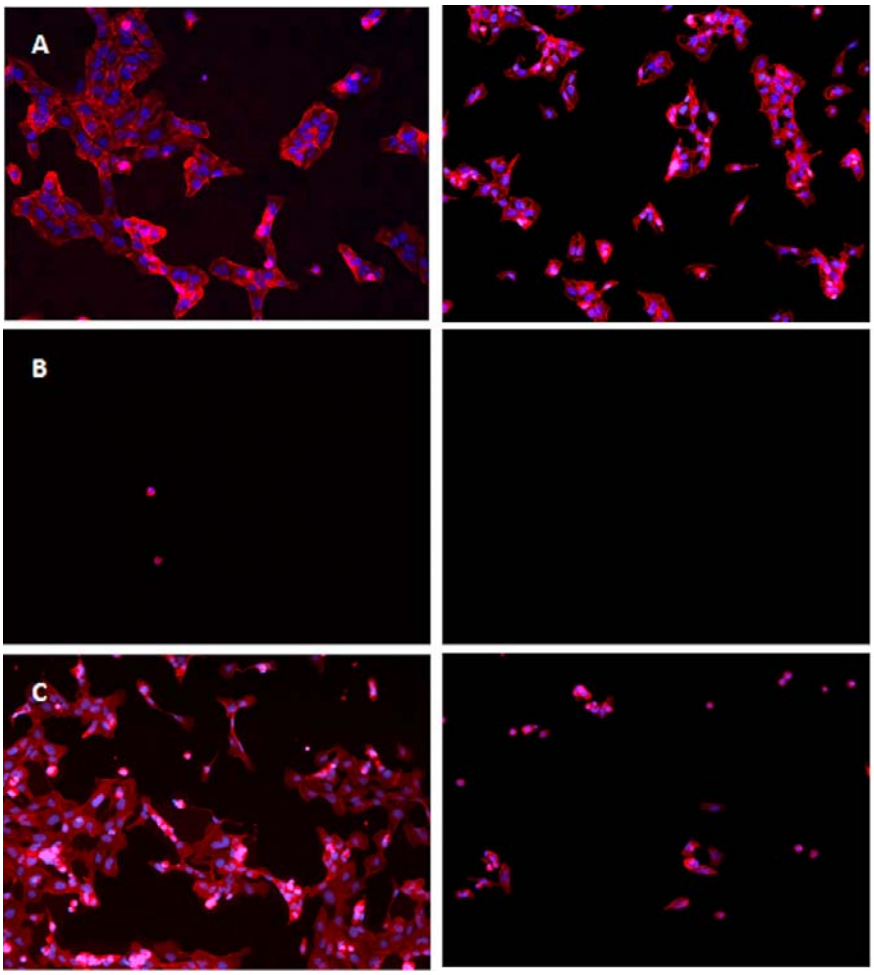

Figure 3. U2OS adhesion and spreading after 48 h to: glass (A), inert OptiChem® (B) and reactive OptiChem® (C), in the absence (left column) or presence (right column) of adherent *Staphylococcus epidermidis* 3399. Scale bar corresponds to 100 µm.

Densities of osteoblast cells present on each substratum surface after 1.5 h were similar for all surfaces (~4000 cell/cm^2), but the percentage area covered by cells varied per substratum. The percentage area covered by adherent U2OS cells after 1.5 h and 48 h is presented in Figure 4. In the absence of co-adhering staphylococci, cells spread more readily on reactive OptiChem® than on inert OptiChem® or glass (see Figure 4A). However, in the presence of adhering co-cultured staphylococci, cell surface coverage was reduced both on glass as well as on NHS-reactive OptiChem®. However, the extent of decrease on the NHS-reactive PEG-based coating approximated the low level of cell coverage observed on the inert PEG-based coating (see Figure 4B).

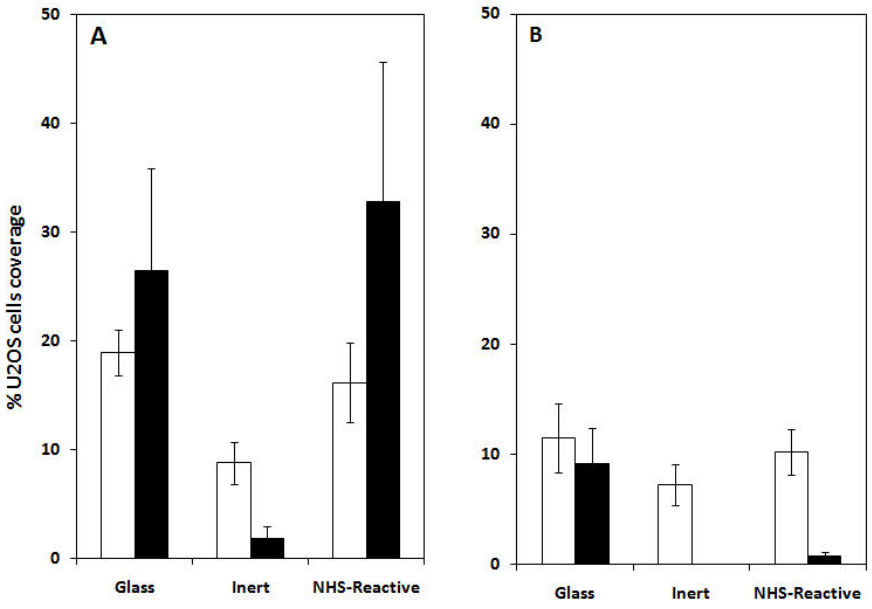

Figure 4. Percentage covered area by U2OS cells in the absence (A) or presence (B) of adhering *S. epidermidis* on glass, inert OptiChem® and reactive OptiChem®, after 1.5 h (□) and 48 h (■). Scale bar corresponds to the standard error over triplicate assays.

Discussion

Competing cell-surface interactions on biomaterial surfaces between opportunistic pathogens and host tissue cells is a critical determinant for the development of biomaterial-associated infections (BAI) and therefore an important design parameter for improving implanted devices. PEG-based coatings are recognized to be very effective

in reducing *in vitro* bacterial adhesion and biofilm formation.[18,21] Therefore, PEG-based coatings have been extensively studied to reduce the risk of BAI.[9,17,18,22,23] In this study, bacterial and tissue cell competitive adhesion and growth in co-culture flow cells were evaluated after 48 h of simultaneous growth on both deactivated 'inert' and NHS-reactive commercial PEG-based coatings. Staphylococcal biofilms on PEG-based coatings were less dense and adhered more weakly than on glass. Furthermore, inert PEG-based coatings did not support osteoblast cell adhesion in optimal media, whereas NHS-reactive PEG-based coatings enhanced mammalian cell adhesion and spreading with respect to inert OptiChem® or uncoated glass. Interestingly, the presence of co-adhered staphylococci notably decreased the ability of U2OS cells to cover all substratum surfaces, also on NHS-reactive PEG-based coatings.

Mammalian cell adhesion to biomaterials surfaces in complex biological milieu depends largely upon cell surface receptors interacting specifically with various extracellular matrix proteins (ECM) adsorbed to substratum surfaces.[24] By contrast, bacteria use both specific and non-specific attachment mechanisms to surfaces. Cells must therefore out-compete bacteria using specific cell-surface interactions in physiologically relevant media, and intrinsically slower proliferation kinetics in order to effectively hinder bacterial colonization of biomaterials. In order to promote better tissue integration, PEG-based coatings are modified with ECM-based peptides and proteins to enhance tissue cell adhesion while simultaneously maintaining anti-adhesive properties against bacteria, known for PEG-based coatings.[11-13] While this conceptual design has been described, co-culture experiments of bacteria and cells to prove their actual efficacy are only infrequently reported. In their reactive form, OptiChem® coatings have amine-reactive esters (NHS) to allow covalent immobilization of peptides and proteins,[14] suggesting covalent interactions with many adhesive proteins from FBS in the "optimal medium" used here, or even directly with cell membrane proteins. Indeed, in the absence of adhering staphylococci, we observed enhanced adhesion, spreading and growth of U2OS cells on NHS-reactive OptiChem® compared to inert OptiChem® or glass. Interestingly, evaluation of PEG-based coatings in the presence of adhering staphylococci during flowing co-culture indicated that favorable effects of the NHS-functionalities on tissue interactions with the coating had disappeared. This suggests

that secretion products (i.e., proteins, glycans) produced by adherent and or growing staphylococci must have a high affinity for reacting with or blocking the NHS-functionality, making these functional groups unavailable for subsequent interactions with host tissue cells. Interestingly, PEG-based coatings functionalized to promote cell interactions using well-known arginine-glycine-aspartic acid (RGD) moieties as a cell-specific integrin-binding peptide did not lose their cell adhesive properties in the presence of adhering staphylococci.[12-13] Loss of osteoblast integrating properties of the NHS-reactive PEG-based coatings in the presence of co-cultured bacteria are therefore unexpected, but these results point to the need for simultaneous co-culture evaluation of bacteria interactions in the presence of host cells, especially when functionalized coatings are involved.[19] Monoculture experiments with OptiChem® surfaces showing (1) reduction in bacterial adhesion and biofilm formation under flow, and (2) enhanced osteoblast adhesion in the presence of culture media containing proteins, lead to the false conclusions that these desirable properties would be maintained in the presence of both adhering species. Clearly, given the inability to control BAI resulting from bacterial interactions with implanted biomaterials *in vivo*, these co-culture experiments are important to provide more accurate and valuable new insights to designing improved, infection-resistant implant materials.

References

1. Kwakman PHS, Te Velde AA, Vandenbroucke-Grauls CMJE, Van Deventer SJH, Zaat SAJ. Treatment and prevention of *Staphylococcus epidermidis* experimental biomaterial-associated infection by bactericidal peptide 2▽ *Antimicrob Agents Chemother* 2006; 50:3977-3983.
2. Lidwell OM, Lowbury EJ, Whyte W, Blowers R, Stanley SJ, Lowe D. Effect of ultraclean air in operating rooms on deep sepsis in the joint after total hip or knee replacement: a randomised study. *Br Med J (Clin Res Ed)* 1982; 285:10-14.
3. Whyte W, Hodgson R, Tinkler J. The importance of airborne bacterial contamination of wounds. *J Hosp Infect* 1982; 3:123-135.
4. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* 1987; 237:1588-1595

5. Everaert EPJM, Van der Mei HC, Busscher HJ. Adhesion of yeasts and bacteria to fluoro-alkylsiloxane layers chemisorbed on silicone rubber. *Coll Surfaces B: Biointerfaces* 1998; 10:179-190.

6. Tsibouklis J, Stone M, Thorpe AA, Graham P, Peters V, Heerlien R, Smith JR, Green KL, Nevell TG. Preventing bacterial adhesion onto surfaces: the low-surface-energy approach. *Biomaterials* 1999; 20:1229-1235.

7. Gottenbos B, Grijpma DW, Van der Mei HC, Feijen J, Busscher HJ. Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria. *J Antimicrob Chemother* 2001; 48:7-13.

8. Tiller JC, Liao CJ, Lewis K, Klibanov AM. Designing surfaces that kill bacteria on contact. *Proc Natl Acad Sci USA* 2001; 98:5981-5985.

9. Roosjen A, Norde W, Van der Mei HC, Busscher HJ. The use of positively charged or low surface free energy coatings versus polymer brushes in controlling biofilm formation. Progr *Colloid Pol Sci* 2006, 132:138-144.

10. Currie EP, Norde W, Cohen SMA. Tethered polymer chains: surface chemistry and their impact on colloidal and surface properties. *Adv Coll Interf Sci* 2003;100:205–65

11. Lavanant L, Klok HA. Biofunctional and biomimetic polymer brushes prepared via surface-initiated atom transfer radical polymerization. *Chimia* 2008; 62:793-798.

12. Maddikeri R, Tosatti S, Schuler M, Chessari S, Textor M, Richards RG, Harris LG. Reduced medical infection related bacterial strains adhesion on bioactive RGD modified titanium surfaces: A first step toward cell selective surfaces. *J Biomed Mater Res A* 2008; 84A:425-435.

13. Shi ZL, Neoh KG, Kang ET, Poh C, Wang W. Bacterial adhesion and osteoblast function on titanium with surface grafted chitosan and immobilized RGD peptide. *J Biomed Mater R* 2008: 86A:865-872.

14. Harbers GM, Emoto K, Greef C, Metzger SW, Woodward HN, Mascali JJ. Functionalized poly(ethylene glycol)-based bioassay surface chemistry that facilitates bio-immobilization and inhibits nonspecific protein, bacterial, and mammalian cell adhesion. *Chem Mater* 2007; 19:4405–4414.

15. Mao G, Metzger S, Lochhead MJ. US patent 6,844,028, 2005.

16. Takahashi H, Emoto K, Dubey M, Castner DG, Grainger DW. Imaging surface immobilization chemistry: correlation with cell patterning on non-adhesive hydrogel thin films. *Adv Funct Mater* 2008; 18:2079–2088.

17. Saldarriaga Fernández IC, Van der Mei HC, Lochhead MJ, Grainger DW, Busscher HJ. The inhibition of the adhesion of clinically isolated bacterial strains on multi-component cross-linked poly(ethylene glycol)-based polymer coatings. *Biomaterials* 2007; 28:4105-12.

18. Saldarriaga Fernández IC, Van der Mei HC, Metzger S, Grainger DW, Engelsman AF, Nejadnik MR, Busscher HJ. *In vitro* and *in vivo* comparisons of staphylococcal biofilm formation on a cross-linked poly(ethylene glycol)-based polymer coating. *Acta Biomater* 2010; 6:1119-1124

19. Subbiahdoss G, Kuijer R, Grijpma DW, Van der Mei HC, Busscher HJ. Microbial biofilm growth vs. tissue integration: "the race for the surface" experimentally studied. *Acta Biomater* 2009; 5:1399-1404.

20. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; 284:1318-1322.

21. Nejadnik MR, van der Mei HC, Norde W, Busscher HJ. Bacterial adhesion and growth on a polymer brush-coating. *Biomaterials* 2008; 29:4117-4121.

22. Kingshott P, Wei J, Bagge-Ravn D, Gadegaard N, Gram L. Covalent attachment of poly(ethylene glycol) to surfaces, critical for reducing bacterial adhesion. *Langmuir* 2003;19:6912–21.

23. Marsh LH, Coke M, Dettmar PW, Ewen RJ, Havler M, Nevell TG. Adsorbed poly(ethyleneoxide)-poly(propyleneoxide) copolymers on synthetic surfaces: spectroscopy and microscopy of polymer structures and effects on adhesion of skin-borne bacteria. *J Biomed Mater Res* 2002;61:641–52.

24. Dewez JL, Doren A, Schneider YJ, Rouxhet PG. Competitive adsorption of proteins: key of the relationship between substratum surface properties and adhesion of epithelial cells. *Biomaterials* 1999; 20:547–559.

Chapter 6

Macrophage Response to Staphylococcal Biofilm on Crosslinked Poly(ethylene glycol)-based Polymer Coatings *In Vitro*

Submitted to Eur cell mater. Isabel C. Saldarriaga Fernández, Joana F. Da Silva Domingues, Theo G. van Kooten, Steven Metzger, David W. Grainger, Henk J. Busscher, Henny C. van der Mei.

Abstract

Biomaterial-associated infections (BAI) are serious clinical complications that threaten the longevity of implanted devices and lead to high morbidity and mortality. Poly(ethylene) glycol (PEG) coatings have been studied as a strategy to reduce the incidence of BAI by reducing protein deposition and the requisite conditioning film that promotes pathogen adhesion and growth on device surfaces. Despite their effectiveness to reduce protein adsorption and a hundred-fold reduction in bacterial adhesion, PEG-based coatings still facilitate weak bacterial adhesion that can form an initial basis for biofilms. Here, we describe a methodology enabling direct, quantitative and detailed qualitative *in situ* observation of macrophage morphology, migration and phagocytosis of bacteria. The methodology was used to compare the *in vitro* interaction of macrophages with *Staphylococcus epidermidis* 3399 adhering to commercial, crosslinked PEG-based coatings (OptiChem®). Adhesion, phagocytosis and migration were studied real-time in a parallel plate flow chamber. Macrophages cultured on OptiChem® coatings showed enhanced migration and phagocytosis of bacteria compared to uncoated glass. Bacterial clearance per macrophage on both inert and reactive OptiChem® coatings was about three times higher than on uncoated glass, corresponding with 70 to 80% reduction in bacterial numbers on OptiChem®, whereas on glass only 20% bacterial reduction was obtained. These findings suggest that bacterial clearance from crosslinked PEG-based coatings by macrophages is more effective than from glass, possibly resulting from weak adhesion of bacteria on OptiChem®. Moreover, macrophages exhibit higher mobility on OptiChem® retaining an improved capability to clear bacteria from larger areas than on glass, where they appear immobilized.

Introduction

Placement of indwelling medical devices into the human body to support and restore function has become common practice in modern medicine with reasonable overall success rates. In 2006, for instance, nearly 800,000 primary total hip and knee arthroplasties were performed solely in the United States.[1] Biomaterial-associated infections (BAI) and adverse interactions between the indwelling device and the surrounding tissues and cells are, however, factors that threaten the device's functionality and longevity.[2] BAI, although of relatively low incidence, represents a serious complication of extensive significance, with related high morbidity and mortality rates, as well as with high associated health care costs. Despite advances in surgical techniques, peri-operative contamination remains the most common route for the infection of biomaterial devices.[3] Microorganisms, usually sourced from the patient's skin, adhere to the implant surface, colonize it and rapidly form biofilms.[4] Generally, microorganisms embedded in biofilms are much less susceptible to antimicrobial treatments[5] and host immune mechanisms than planktonic organisms and hence, infection usually persists until the device is removed.

BAI pathogenesis depends on many factors, such as implant site, device type and the patient's general health status, but also on the interaction between the biomaterial surface, the host's immune system and the infecting pathogen.[1,6] Following biomaterial implantation, tissue trauma and injury trigger a cascade of physiological events that activate the immune system.[7] Neutrophils and monocytes/macrophages are the major host inflammatory cell populations that arrive within minutes to hours at the implant site.[7-8] In contrast to neutrophils that may disappear within hours, macrophages increase in numbers over time and remain at the implant surface for several weeks depending on the severity of the injury.[8] Macrophages orchestrate the host inflammation process and eventual foreign body reactions, but are also an important active component in pathogen clearance. During bacterial infection *in vivo*, macrophages adhere to the infected tissue and detect bacteria via cell surface receptors.[7] After recognition and pathogen attachment, macrophages engulf bacteria (phagocytosis) and activate cellular functions such as proliferation, secretion of proteins and cytokines, and respiratory burst to

destroy phagocyted bacteria and recruit other cells from the adaptive immune system, as for example T and B lymphocytes.[9] However, the presence of a biomaterial affects the immune system's response to bacterial infection, and the biomaterial surface chemistry can stimulate[7] or reduce macrophage adhesion, phagocytic activity and migration.[10-11] Therefore, macrophage-biomaterial-bacteria interactions are crucial factors influencing pathogenesis of BAI.[11]

Poly(ethylene) glycol (PEG) has been promoted as an infection-resistant biomaterial coating due to low protein adsorption and hundred-fold reductions in adhering bacteria with respect to common biomaterials.[12-15] Nonetheless, this performance does not prevent the formation of weakly adhering, more mature biofilms.[12,16] Currently, it is unknown how macrophages deal with the few bacteria adhering to PEG-based coatings. Therefore, the aim of this study was to investigate the *in vitro* response of macrophages to *Staphylococcus epidermidis* adhering to a fully characterized commercial, multi-component crosslinked PEG-based polymer coating (OptiChem®, Accelr8 Technology Corp. USA).

Materials and Methods

Substrata

OptiChem®-coated glass slides (Accelr8 Technology, USA, now commercially available as Schott-Nexterion™ Slide H) were supplied by Accelr8 Technology Corporation (Denver, USA). OptiChem® is a multi-component, crosslinked transparent and robust polymer coating, having PEG as its active component. The surface coating has an amine-reactive (i.e. an NHS active ester) terminal chemical functionality to allow specific immobilization of biomolecules. The NHS chemistry can also be deactivated to provide a surface with very low, nonspecific binding of biological.[17-19] Extensive surface chemistry and analytical details regarding the coating and its bio-immobilization properties have been published.[17-19]

OptiChem® was applied on optical–grade glass slides by spin coating and curing. Slides were stored at -20°C until use. Half of the coated slides were deactivated by quenching

the NHS surface groups ("inert OptiChem®") using hydroxyethylamine.[17] The remaining slides were used in its NHS-reactive form, denoted here as "reactive OptiChem®". Glass was used as a control surface. Glass slides were cleaned in 2% RBS 35 detergent solution (Omnilabo International BV, Breda, The Netherlands) under sonication and rinsed with demineralized water, submerged in methanol, washed with water again and finally with demineralized water. All samples were sterilized in 70% ethanol for 10 min and rinsed with sterile, demineralized water and finally with sterile phosphate buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 6.8).

Bacterial strain and growth conditions

S. epidermidis 3399 is a clinical isolate from the skin and was used because skin-derived organisms like *S. epidermidis* are often involved in peri-operative contamination of biomaterial implant surfaces. The staphylococcus was first grown aerobically overnight at 37°C on blood agar plates from a frozen stock. The plates were kept at 4°C, never longer than 2 weeks. One colony was used to inoculate 10 ml of tryptone soya broth (TSB, OXOID, Basingstoke, England), which was incubated for 24 h at 37°C and used to inoculate a second culture in 200 ml TSB. Bacteria were harvested after overnight growth by centrifugation (5 min at 5000 g at 10°C) and washed twice with sterile PBS. Bacteria were resuspended in sterile PBS to a concentration of 3×10^8 bacteria ml^{-1}.

Cell culture conditions

J774 mouse macrophages were grown in tissue culture polystyrene (TCPS) flasks (Greiner, Germany), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5g l^{-1} D-glucose, pyruvate, and 10% fetal bovine serum (DMEM + 10% FBS) at 37°C in a humidified atmosphere of 5% CO_2. Cells were passaged every four days at 70-80% confluency by scraping. The cells were passaged up to a maximum of seven times.

J774 morphology, migration and phagocytic activity

Macrophage morphology, migration and phagocytic activity on OptiChem® coatings were assessed using real-time *in situ* image analysis in a parallel plate flow chamber with

a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope (Olympus BH-2) (for a detailed description of the system, see Busscher *et al.*[20]). Assays were performed on the bottom plate of the flow chamber containing the substrata under study. The system was first filled with sterile PBS to remove air-bubbles from the tubing and chamber, and perfused for 30 min with a laminar flow of 1.5 ml min^{-1}, corresponding to a wall shear rate of 11 s^{-1}. Then, flow was switched to bacterial suspension in PBS that circulated at the same flow rate until the density of adhering bacteria had reached 4.7 x 10^5 bacteria/cm^2 on all substrata, as evaluated real-time with the image analysis system. Subsequently, the suspension was switched once more to sterile PBS to remove unbound bacteria from the system. The flow chamber was warmed up to 37°C. Then, a macrophage suspension consisting of 7.5 x 10^5 cells ml^{-1} in DMEM + 10% FBS was introduced into the system. Once the entire volume of buffer inside the chamber was replaced by the cell suspension, flow was stopped. Images were collected throughout the assay for 120 min at 1 min intervals. Phagocytic activity was determined by comparing the number of bacteria adhering per cm^2 on the substrata at different time intervals. In addition, the difference between the initial numbers of bacteria adhering to the substratum prior to exposure to macrophages and the final bacterial density after 120 min exposure to the macrophages was calculated to determine the number of bacteria ingested per adherent macrophage. Bacterial growth during 120 min phagocytic activity was minimal in DMEM + 10% FBS and therefore neglected in these calculations.

Results

Macrophage morphology

Phase contrast images of cultured J774 murine macrophages interacting with bacteria adhering to glass, inert and reactive OptiChem® in DMEM + 10% FBS are shown in Figure 1. Macrophages adhering to glass maintain a spherical shape throughout the experiment, while those interacting with inert and reactive OptiChem® acquire a more elongated form increasing the contact area with the surface.

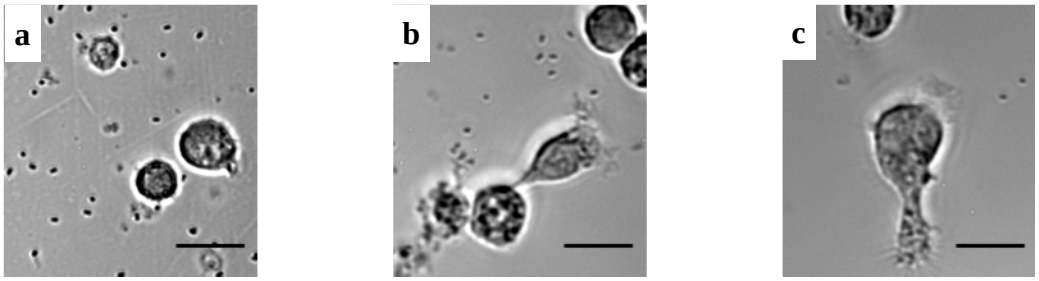

Figure 1. Phase-contrast microscopic images showing the different morphologies of macrophages adhering to different substrata in the presence of *S. epidermidis* 3399 in serum-containing culture media in the flow chamber. (a) glass, (b) inert OptiChem®, and (c) reactive OptiChem®. The bar denotes 20 µm.

Macrophage migration and phagocytic activity

Macrophage activity was assessed microscopically in real-time. The number of macrophages adhering per cm^2 on each substratum is presented in Table 1. J774 cell migration and phagocytosis of bacteria on glass, inert and reactive OptiChem® in the presence of adhering staphylococci are shown in Figure 2. Macrophages adhering to glass are immobilized to the substratum and their migration is restricted to a few µm's. Consequently, macrophages only phagocytose bacteria attached in their close surroundings via the projection of pseudopodia. In contrast, macrophages adhering on inert and reactive OptiChem® coatings are more mobile, migrating relatively freely over the substratum towards adherent staphylococci.

Table 1. Numbers of *S. epidermidis* remaining adherent on the surface per unit surface area after exposure to macrophages (N_{2h}) for 120 min, together with the numbers of macrophages per unit surface area and the number of staphylococci taken per macrophage for the three substrata involved in this study. The number of adhering staphylococci prior to exposure to macrophages was $4.7 \pm 0.9 \times 10^5\ cm^{-2}$, as determined during an experiment using real-time *in situ* observation. SD over six images per substratum surface.

Substratum	N_{2h} ($10^5/cm^2$)	Macrophages ($10^4/cm^2$)	Bacteria/macrophage
Glass	4.1 ± 0.3	5.2 ± 0.8	2.0 ± 0.4
Inert OptiChem®	1.4 ± 0.3	4.3 ± 0.5	6.7 ± 1.1
Reactive OptiChem®	1.1 ± 0.3	5.6 ± 0.2	6.1 ± 0.5

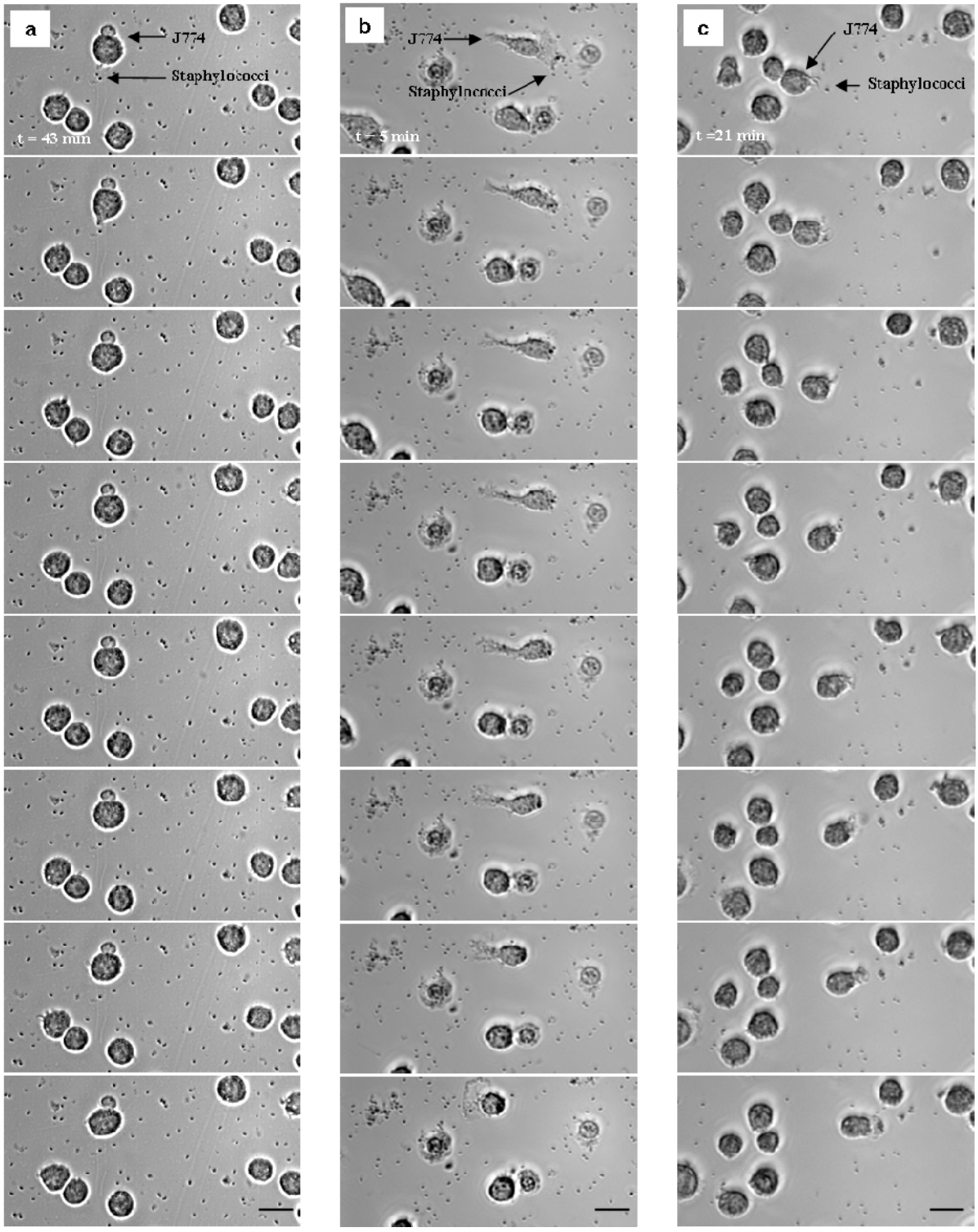

Figure 2. Time-lapse light micrographs of the migration and phagocytosis of *S. epidermidis* 3399 by murine macrophages in serum-containing culture media in the flow chamber on (a) glass, (b) inert OptiChem®, and (c) reactive OptiChem®. The interval between the micrographs is 2 min, increasing from top to bottom. "t" denotes the time of exposure to macrophages. The bar denotes 20 µm. See supplementary information for video time-lapse files of macrophage real-time migration and phagocytosis.

Under sterile operating conditions, the number of bacteria-carrying particles that fall on an open wound varies between 10^2 and 10^5 per cm^2.[21-23] In this study, the bacterial density on all substrata was 4.7 ± 0.9 x 10^5 per cm^2 before macrophages were added into the system. The number of bacteria on the surface can thus be considered reasonably close to a clinically relevant situation of peri-operative contamination. After exposure to macrophages, the numbers of adhering staphylococci decreased significantly. Figure 3 shows the percentage of bacteria left adhering on the surface as a function of exposure time to macrophages. Bacterial clearance per macrophage on OptiChem® coatings was nearly three times higher than on the control surface, irrespective of whether the substrate was an inert or reactive OptiChem® coating (see Table 1).

Bacterial clearance per macrophage on OptiChem® coatings was nearly three times higher than on the control surface, irrespective of whether the substrate was inert or reactive OptiChem® coating (see Table 1).

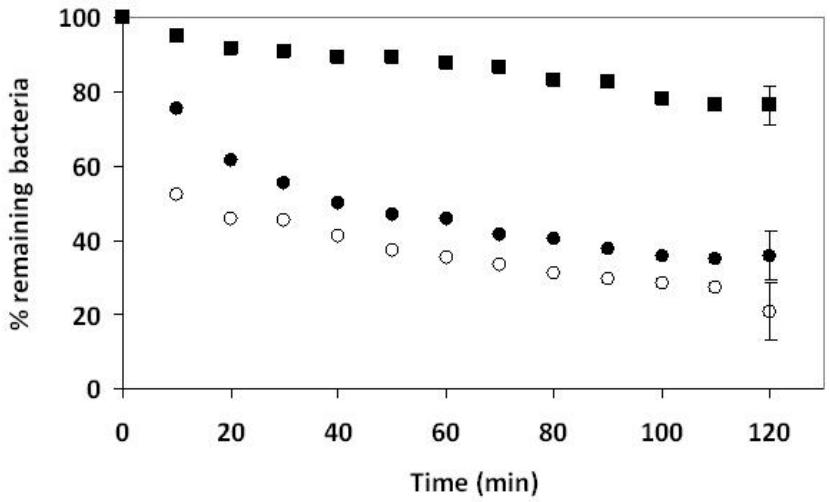

Figure 3. Percentage of adhering *S. epidermidis* 3399 remaining on the various surfaces after exposure to macrophages for 120 min in serum-containing cell culture media with respect to their initial adherent density (4.7 x 10^5 cm^{-2}) on: glass (■), inert OptiChem® (○), and reactive OptiChem® (●). Error bars represent the standard deviation over six images.

Discussion

Macrophages are primary infiltrating immune system cells responding rapidly to wounding and implanted biomaterials, and are directly involved in the host inflammatory and foreign body response as well as in the defense against infectious pathogens. Macrophages adhere to device surfaces and remain at the implant-tissue interface for several days to realize their functions. Hence, the interaction between macrophages and bacterially contaminated biomaterials is crucial in the development of BAI.[8,11] A mature biofilm is less likely to form if macrophages are able to remove and destroy microorganisms adhering on an implanted device. The response of macrophages to surfaces modified with PEG-based coatings has been assessed by others,[24-26] but never on bacterially contaminated biomaterial surfaces as done here. Our study showed that macrophages phagocytosis of bacteria adhering on inert and reactive OptiChem® was similar for both surfaces but approximately three times higher than on uncoated glass. This difference and elevated phagocytic activity of macrophages to *S. epidermidis* adhering on crosslinked PEG-based coatings is attributed to an almost unlimited macrophage mobility on the PEG-based coating compared to glass. On OptiChem®, macrophages reduced the numbers of adhering staphylococci by approximately 80% over a 2 h time period, as shown in Figure 3. There are no comparative data available in the literature to determine whether this is a high or low phagocytosis efficiency. In a recent study phagocytosis of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on PEG-*graft*-polyacrylate (PEG-*g*-PA) co-polymers has been studied.[27] However, macrophages were allowed to adhere to the surface before bacteria were incorporated into the system, which is an entirely different model situation than our peri-operative model.

The enhanced macrophage mobility and phagocytic activity on OptiChem® coatings could result from weak cell-surface interactions between these cells and the PEG-based coatings. In this response, both macrophage-surface and bacteria-surface adhesion forces are important for this analysis. Adhesion forces between microorganisms and poly(ethylene) oxide (PEO) brush coatings have been assessed using atomic force microscopy and found to be up to 10 times smaller for various *Pseudomonas aeruginosa*

strains on a PEO brush than on bare glass.[28] Incremental increases in shear rate in a parallel plate flow chamber also indicated that the adhesion strength of *S. epidermidis* and *Staphylococcus aureus* is decreased on PEO-coated silicone rubber. More than 85% of these bacteria could be sheared off from the PEO brush coating whereas up to 10% of adherent bacteria could be stimulated to detach from pristine silicone rubber.[13] Analogous to bacterial interactions with polymer brush coatings, macrophages adhering to OptiChem® coatings may be expected to experience weak adhesion forces as well, allowing them to move freely over the substratum towards adhering bacteria. Low adsorption of serum proteins on PEG-based surfaces, and specifically for OptiChem® coatings[17] produces poor cell adhesion.[17,19] Macrophage-surface interactions depend less on cell matrix-type adhesive proteins in contrast to other cell types,[29] and macrophage surface mobility is increased without a substantial surface-adsorbed protein layer. This occurs on both the inactivated (inert) PEG surface as well as that retaining the NHS-reactive immobilizing chemistry. Weak interactions between adhering bacteria and OptiChem® coatings, as described above, may also help facilitate more efficient macrophage phagocytosis from these surfaces. This is an advantage, as phagocyte–mediated clearance of surface-adhered bacteria is more difficult for macrophages than their clearance of planktonic bacteria.[30]

In vivo, the interaction between proteins, pathogens and the host defense cells at the biomaterial-tissue interface is a complex process where each may contribute to bacterial survival and persistence on biomaterials and in adjacent tissues.[2,11] Host defense functions are suggested to be affected in the presence of an infected biomaterial, for example, by diminishing host phagocytic.[10,11,31] We demonstrate that macrophages can phagocytose adhering bacteria more effectively on PEG-based coatings. Although macrophages are not the only cell type present at the interface *in vivo*, these results for macrophages are relevant, in that macrophages remain at the implanted biomaterial surface for longer periods of time than other cells.[8] Also an important factor in the persistence of BAI is bacterial survival within macrophages once ingested. This intra-phagocyte survival mechanism is both pathogen and substratum-dependent.[11] That such bacterial survival within macrophages is favored on OptiChem® coatings was not the focus of this study and should be elucidated.

Conclusions

We introduced a novel *in vitro* methodology to enable direct, quantitative and detailed qualitative *in situ* observations of macrophage adherent morphology, migration and engulfment of surface-resident bacteria. In the current study, we employed this methodology to compare macrophage clearance of adhering staphylococci from glass and commercial, crosslinked PEG-based coatings. Substratum surfaces were first contaminated with bacteria prior to exposure to cultured macrophages in serum-based media to mimic peri-operative bacterial contamination conditions. Macrophages on crosslinked PEG-based coatings exhibited enhanced cell mobility compared to the glass surface, likely due to weak cell-surface interaction forces arising from strongly hydrated, low protein-adsorbing crosslinked PEG-based coatings. This greater intrinsic cell mobility and associated weak bacterial-surface adhesion forces facilitated higher phagocytosis on the PEG surfaces. Macrophage-mediated bacterial clearance was about three times more effective on the multi-component crosslinked PEG-based coatings (OptiChem®) than on glass, irrespective whether the surface was the inactivated or reactive NHS-derivatized PEG-based coating.

References

1. Del Pozo JL, Patel R. Infection associated with prosthetic joints. *N Engl J Med* 2009; 361: 787-794.

2. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* 1987; 237:1588-1595.

3. Busscher HJ, Ploeg RJ, Van der Mei HC . Snapshot: biofilms and biomaterials; mechanisms of medical device related infections. *Biomaterials* 2009; 25:4247-4248.

4. Knobben BAS, Engelsma Y, Neut D, Van der Mei HC, Busscher HJ, Van Horn JR. Intraoperative contamination influences wound discharge and periprosthetic infection. *Clin Orthop Relat Res* 2006: 452:236-241.

5. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet* 2001; 358:135-138.

6. Kwakman PH, Te Velde AA, Vandenbroucke-Grauls CM, Van Deventer SJ, Zaat SA. Treatment and prevention of *Staphylococcus epidermidis* experimental biomaterial-associated infection by bactericidal peptide 2. *Antimicrob Agents Chemother* 2006; 50:3977-3983.

7. Xia Z, Triffitt JT. A review on macrophage responses to biomaterials. *Biomed Mater* 2006; 1: R1-9.

8. Anderson JM. Biomaterials Science. An Introduction to Materials in Medicine. 2004. Elsevier San Diego, CA, pp 296-304.

9. Berton G, Lowell CA. Integrin signalling in neuthrophils and macrophages. *Cell Signal* 1999; 11:621-635.

10. Henke PK, Bergamini TM, Brittian KR, Polk HC Jr. Prostaglandin E2 modulates monocyte MHC-II (Ia) suppression in biomaterial infection. *J Surg Res* 1997; 69:372-378.

11. Boelens JJ, Dankert J, Murk JL, Weening JJ, van der Poll T, Dingemans KP, Koole L, Laman JD, Zaat SAJ. Biomaterial-associated persistence of *Staphylococcus epidermidis* in pericatheter macrophages. *J Infect Dis* 2000; 181:1337-1349.

12. Nejadnik MR, Van der Mei HC, Norde W, Busscher HJ. Bacterial adhesion and growth on a polymer brush-coating. *Biomaterials* 2008; 29:4117-4121.

13. Nejadnik MR, Van der Mei HC, Busscher HJ, Norde W. Determination of the shear force at the balance between bacterial attachment and detachment in weak-adherence systems, using a flow displacement chamber. *Appl Environ Microbiol* 2008;74:916-919.

14. Norde W, Gage D. Interaction of bovine serum albumin and human blood plasma with PEO-tethered surfaces: influence of PEO chain length, grafting density, and temperature. *Langmuir* 2004; 20:4162–4167.

15. Saldarriaga Fernández IC, Van der Mei HC, Lochhead MJ, Grainger DW, Busscher HJ. The inhibition of the adhesion of clinically isolated bacterial strains on multi-component cross-linked poly(ethylene glycol)-based polymer coatings. *Biomaterials* 2007; 28:4105-4112.

16. Saldarriaga Fernández IC, Van der Mei HC, Metzger S, Grainger DW, Engelsman AF, Nejadnik MR, Busscher HJ. *In vitro* and *in vivo* comparisons of staphylococcal biofilm formation on a cross-linked poly(ethylene glycol)-based polymer coating. *Acta Biomater* 2010; 6:1119-11124.

17. Harbers GM, Emoto K, Greef C, Metzger SW, Woodward HN, Mascali JJ, Grainger DW, Lochhead MJ. Functionalized poly(ethylene glycol)-based bioassay surface chemistry that facilitates bio-immobilization and inhibits nonspecific protein, bacterial, and mammalian cell adhesion. *Chem Mater* 2007; 19:4405–4414.

18. Mao G, Metzger S, Lochhead MJ. US patent 6,844,028, 2005.

19. Takahashi H, Emoto K, Dubey M, Castner DG, Grainger DW. Imaging surface immobilization chemistry: correlation with cell patterning on non-adhesive hydrogel thin films. *Adv Funct Mater* 2008; 18:2079–2088.

20. Busscher HJ, Van der Mei HC . Microbial adhesion in flow displacement systems. *Clin Microbiol Rev* 2006; 19:127-141.

21. Fitzgerald RH. Microbiologic environment of the conventional operating-room. *Coll Surf B Biointerfaces* 1979; 19:127-141.

22. Oakes JA, Wood AJJ. Infections in surgery. *N Engl J* 1986; 315:1129–1138.

23. Nelson CL. Environmental bacteriology in the unidirectional (horizontal) operating room. *Arch Surg* 1979; 114:778–782.

24. Lynn AD, Kyriakides TR, Bryant SJ. Characterization of the *in vitro* macrophage response and *in vivo* host response to poly(ethylene glycol)-based hydrogels. *J Biomed Mater Res A* 2010; 93:941-953.

25. Schmidt DR, Kao WJ. Monocyte activation in response to polyethylene glycol hydrogels grafted with RGD and PHSRN separated by interpositional spacers of various lengths. *J Biomed Mater Res A* 2007; 83:617-625.

26. Jenney CR, Anderson JM. Effects of surface-coupled polyethylene oxide on human macrophage adhesion and foreign body giant cell formation *in vitro*. *J Biomed Mater Res* 1999; 44:206-216.

27. Wagner VE, Bryers JD. Poly(ethylene glycol)-polyacrylate copolymers modified to control adherent monocyte-macrophage physiology: interactions with attaching *Staphylococcus epidermidis* or *Pseudomonas aeruginosa* bacteria. *J Biomed Mater Res A* 2004; 69:79-90.

28. Roosjen A, Busscher HJ, Norde W, Van der Mei HC. Bacterial factors influencing adhesion of *Pseudomonas aeruginosa* strains to a poly(ethylene oxide) brush. *Microbiology* 2006; 152:2673-2682.

29. Godek ML, Michel R, Chamberlain LM, Castner DG, Grainger DW. Adsorbed serum albumin is permissive to macrophage attachment to perfluorocarbon polymer surfaces in culture. *J Biomed Mater Res A* 2009; 88:503-519.

30. Leid JG. Bacterial Biofilms Resist Key Host Defenses. *Microbe* 2009; 4:66-70.

31. Kaplan SS, Basford RE, Mora E, Jeong MH, Simmons RL. Biomaterial-induced alterations of neutrophil superoxide production. *J Biomed Mater Res* 1992; 26:1039-1051.

Chapter 7

A New Method to Study the Simultaneous Interaction between Bacteria, Macrophages and Osteoblasts on a Biomaterial Implant Surface

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Abstract

Biomaterial-associated infections (BAI) represent a major clinical problem with considerable consequences in terms of medical care and health care expenditure. The pathogenesis of BAI depends on various factors, such as the bacterial virulence, physico-chemical properties of biomaterial surface and the influence of host cells. The ultimate fate of a biomaterial implant is determined by the outcome of the race for the surface between bacteria and tissue cells. Although models exist to study the simultaneous interaction between bacteria and tissue cells on an implant surface, no model is available that includes macrophages as well. Therefore, in this study, we describe a method to study the simultaneous interaction between bacteria, macrophages and osteoblasts on a biomaterial implant surface. Bacteria were first deposited to a poly(methyl metacrylate) surface to mimic peri-operative contamination, after which osteoblasts and macrophages were seeded. Subsequently, bacteria, osteoblasts and macrophages were allowed to grow simultaneously on the surface for 24 h under low shear (0.14 s^{-1}). Macrophages delayed biofilm growth up to 20 h for *Staphylococcus epidermidis* and up to 14 h for *Staphylococcus aureus*. *S. aureus* biofilms induced death of osteoblasts whereas osteoblast adhesion and spreading was almost unaffected by *S. epidermidis* biofilms, irrespective of macrophage presence in the system. The method outlined in this study provides an important bridge between *in vitro* and *in vivo* experiments for the evaluation of BAI.

Introduction

Biomaterial-associated infections (BAI) are a widespread complication that threatens the longevity and functionality of indwelling biomaterial implants and devices and the consequences in terms of medical care are severe. Despite of improved techniques and highly sterile conditions in the operating theatre, peri-operative contamination by microorganisms suspended in the air and from the skin flora continues to be the most common pathway for the contamination of biomaterial implants and devices.[1,2]

Staphylococcus aureus and *Staphylococcus epidermidis* are microorganisms frequently isolated from BAI. *S. epidermidis* is found in almost 50% of the infections associated with catheters, artificial joints and heart valves, while *S. aureus* is seen in around 23% of the infections associated with prosthetic joints.[3] These commensals from the skin adhere to the biomaterial surface and grow to form a biofilm. Bacteria in their biofilm mode of growth are frequently more resistant to antibiotic treatment and the host immune system than their planktonic counterparts. Hence, removal of an infected implant or device is often the only remedy for a BAI. Surrounding tissue, however, may remain compromised by bacterial presence for prolonged periods of time after removal of the biomaterial,[4,5] which severely lowers the prospects of a secondary implant or device, since bacteria in tissue constitute a new source for BAI to develop.

Whether or not BAI will occur, ultimately depends on the interaction between the biomaterial, the bacterium involved and host cells. Host cells and bacteria battle to proliferate and colonize a biomaterial surface. The general assumption is that on surfaces with a high affinity for tissue cells, bacterial biofilm formation will be limited, thus decreasing the risk of a BAI. Contrary, if bacteria grow and colonize faster than tissue cells, bacterial toxins and virulence factors can impair cell functions leading to BAI.[6]

In a healthy host, the host immune system comes to the aid of tissue cells.[7] Macrophages are one of the most predominant immune cells that arrive within minutes to hours at an implant site and can remain at a biomaterial surface for several weeks to orchestrate the inflammation process and eventually foreign body reactions.[7] During infection, macrophages detect bacteria via cell surface receptors that bind to bacterial ligands and

opsonines.[8] Subsequently, macrophages ingest pathogens and activate cellular functions such as proliferation, secretion of proteins and cytokines, and respiratory burst to destroy phagocyted microorganisms and recruit other cells from the adaptive immune system.[9] However, it has been shown that the presence of a foreign body may impair the host immune system and very low numbers of adherent bacteria are already sufficient to create a BAI.[6]

Currently, biomaterials research is strongly focused on the design of novel functional coatings that reduce the risk of BAI by inhibiting bacterial adhesion and stimulating tissue cell adhesion.[10,11] However, in the evaluation of these coatings, bacterial and tissue cell adhesion are often considered as independent phenomena and not as simultaneous events.[10,11] Furthermore, the response of the immune system to bacterial colonization on these coatings is generally ignored. Recently, a novel *in vitro* method has been forwarded to determine the influence of bacterial presence on the outcome of the competition between bacteria and tissue cells in their attempt to colonize or integrate a biomaterial surface.[12]

In this study we present an extension of this model[12] to include also the influence of macrophages on the outcome of the race for the surface between adhering bacteria and tissue cells in a single experiment.

Materials and methods

Biomaterial

Poly (methyl methacrylate) (PMMA) (Vink Kunststoffen, Didam, The Netherlands) was used as a substratum. Samples were rinsed thoroughly with 70% ethanol (Merck, Darmstadt, Germany) and washed with sterile ultrapure water before use. Water contact angles on thus cleaned PMMA were 73 ± 3 degrees, in line with literature.[13]

Tissue cell culturing and harvesting

U2OS osteosarcoma cells were routinely cultured in Dulbecco's modified Eagles Medium (DMEM)-low glucose supplemented with 10% fetal calf serum (FBS, non-heat

inactivated), 0.2 mM of ascorbic acid-2-phosphate (AA2P). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, and passaged at 70 – 90% confluency using trypsin/EDTA.

Macrophages culturing and harvesting

J774 murine macrophages were routinely cultured in Dulbecco's modified Eagles Medium (DMEM)-high glucose supplemented with 10% fetal calf serum (FBS, non-heat inactivated) and denoted in the paper as "optimal medium". Macrophages were maintained at 37°C in a humidified atmosphere with 5% CO_2, and passaged at 70 – 80% confluency by scraping.

Bacterial growth conditions and harvesting

The bacterial strains used in this study were *S. epidermidis* ATCC 35983 and *S. aureus* ATCC 12600. These strains were cultured on blood agar plates and grown aerobically overnight at 37°C, and the plates were kept at 4°C, never longer than two weeks. For each experiment, one colony was used to inoculate 10 ml of tryptone soya broth (TSB, OXOID, Basingstoke, England) and incubated for 24 h at 37°C in ambient air. This culture was used to inoculate another culture in 200 ml TSB that was incubated overnight at 37°C prior to harvesting. Bacteria were harvested by centrifugation (5 min at 5000 g at 10°C) and washed twice with sterile ultrapure water. Bacteria were sonicated intermittently on ice (30 s) in sterile phosphate buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 6.8) to break bacterial aggregates, and resuspended in 200 ml sterile PBS to a concentration of 3 x 10^6 bacteria per ml. Prior to the experiments, growth and biofilm formation of *S. epidermidis* ATCC 35983 and *S. aureus* ATCC 12600 in optimal medium was confirmed by culturing bacteria in optimal medium for 24 h.

In vitro *multiple cell type culture assays*

The competition between bacteria and U2OS cells for the colonization of PMMA in the absence of macrophages (control) and in the presence of macrophages was assessed under laminar flow on the bottom plate of a parallel plate flow chamber (175 x 17 x 0.75 mm^3).

Bacterial and cell deposition were observed real-time with a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope Olympus BH-2 (Olympus, Germany). The flow chamber was equipped with heating elements and kept at 37°C throughout the experiments.

Prior to each experiment, all tubes and the flow chamber were filled with sterile PBS, taking care to remove all air bubbles from the system. Once the system was filled, and before the addition of bacterial suspension, PBS was allowed to flow through the system at a shear rate of 11 s^{-1}. Then, a bacterial suspension in PBS was perfused through the chamber at the same shear rate and phase-contrast images were obtained. As soon as the desired density of adhering bacteria (10^3 cm^{-2}), was reached, flow was switched to sterile PBS to remove unbound bacteria and the bacterial suspension from the tubes and chamber. Subsequently, a cell suspension consisting of U2OS cells (6×10^5 cells ml^{-1}) and J774 macrophages (12×10^5 cells ml^{-1}) in optimal medium was added to the flow chamber. Once the entire volume of buffer inside the chamber was replaced by the cell suspension, flow was stopped for 1.5 h in order to allow U2OS cells and macrophages to adhere and spread on the substratum surface. Ultimately, optimal medium supplemented with 2% HEPES was perfused through the system without recirculation at a shear rate of 0.14 s^{-1} for 24 h and phase-contrast images were obtained continuously at 2 min intervals. Biofilm growth was assessed in real-time by determining the numbers of adhering bacteria per unit area using proprietary software based on the Matlab Image processing Toolkit (The MathWorks, MA, USA).

At the end of the assay, surfaces were prepared for qualitative analysis to assess U2OS cell and macrophages morphology and spreading. Cells adhering to PMMA were fixed with citrated-acetone-formaldehyde fixative solution for 30 s and stained with an alkaline-dye mixture (Sigma-Aldrich, Germany) (Naphtol AS-BI phosphate, sodium nitrite, fast blue BB base) for 15 min. The samples were subsequently rinsed with demineralized water and counterstained for 2 min with neutral red solution. Then the samples were rinsed once again with demineralized water, allowed to dry and phase-contrast images were taken on different places of the sample. Differentiated U2OS osteosarcoma cells stained purple/blue (alkaline phosphatase-positive) and macrophages were orange stained.

Results

Bacteria were allowed to adhere to the biomaterial surface prior to U2OS cell and macrophage adhesion, mimicking a peri-operative contamination after which bacteria, U2OS cells and macrophages were allowed to grow simultaneously for 24 h. Events are illustrated as follows.

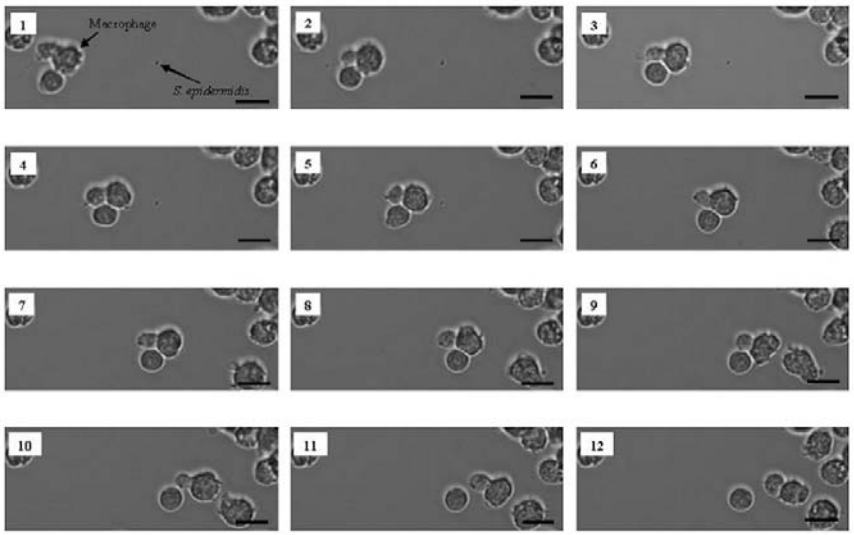

Figure 1. Phase-contrast images of macrophage activity toward *S. epidermidis* ATCC 35983 on a PMMA surface in the presence of U2OS cells: macrophage migration towards *S. epidermidis* (images 1-5), bacterial clearance by phagocytosis (images 6-7) and further migration (images 8-12). The bar denotes 50 µm.

Migration of macrophages towards bacteria and phagocytosis

The number of bacteria adhering to the PMMA surface prior to U2OS cells and macrophages adhesion was set to 10^3 cm^{-2}, using the image analysis system. Subsequently, U2OS cells and macrophages were allowed to adhere to the surface and the simultaneous interactions of bacteria, macrophages and U2OS cells were observed by phase-contrast microscopy. Figure 1 shows macrophage migration in the presence of U2OS cells towards adhering bacteria and subsequent phagocytosis. Macrophage migration towards bacteria and phagocytosis was similar on PMMA colonized by *S. epidermidis* and *S. aureus*.

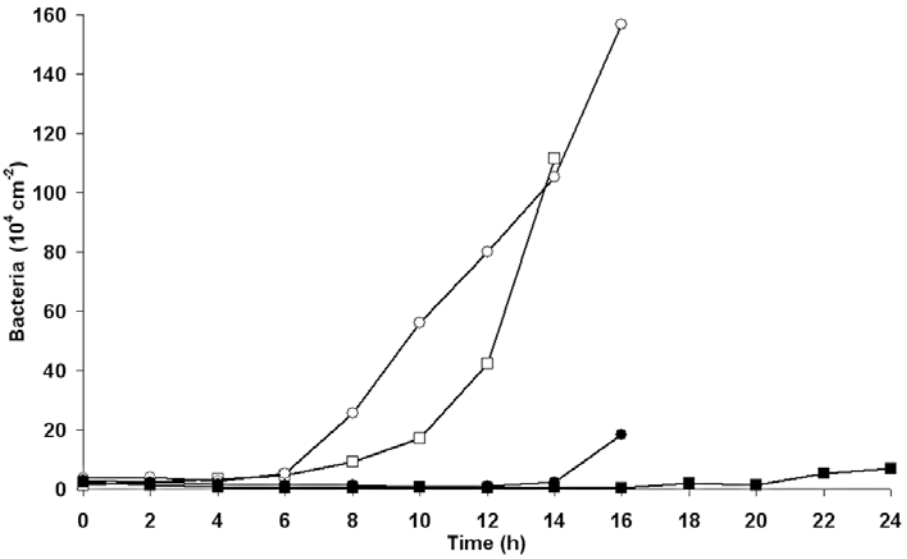

Figure 2. The numbers of adhering bacteria on PMMA as a function of time during the simultaneous growth of bacteria and U2OS cells in the absence and presence of macrophages in a parallel plate flow chamber (shear rate 0.14 s^{-1}). *S. epidermidis* in the absence of macrophages (□), *S. aureus* in the absence of macrophages (○), *S. epidermidis* in the presence of macrophages (■) and, *S. aureus* in the presence of macrophages (●).

Bacterial biofilm formation in the absence and presence of macrophages

Biofilm growth was assessed over time by determining the numbers of bacteria adhering to PMMA at different time points during the simultaneous growth of bacteria, U2OS cells and macrophages (Figure 2). In the presence of macrophages, reduction in the numbers of adherent bacteria, for both S. epidermidis and S*. aureus*, was observed as compared to controls (absence of macrophages). This effect was observed up to 20 h of growth for *S. epidermidis* and up to 14 h for *S. aureus*. Thereafter macrophage burst and release of ingested bacteria was observed.

Bacterial-tissue cell interactions in the absence and presence of macrophages. Immediately after seeding, U2OS cell adhesion and spreading on PMMA was observed independently of whether macrophages were present or not. After 24 h of simultaneous growth, U2OS cell death was observed in the presence of a *S. aureus* biofilm irrespective of the absence or presence of macrophages. On the other hand, colonizing *S. epidermidis* did not significantly affect U2OS cells and their adhesion and spreading were similar both in the absence and in presence of macrophages (see Figure 3).

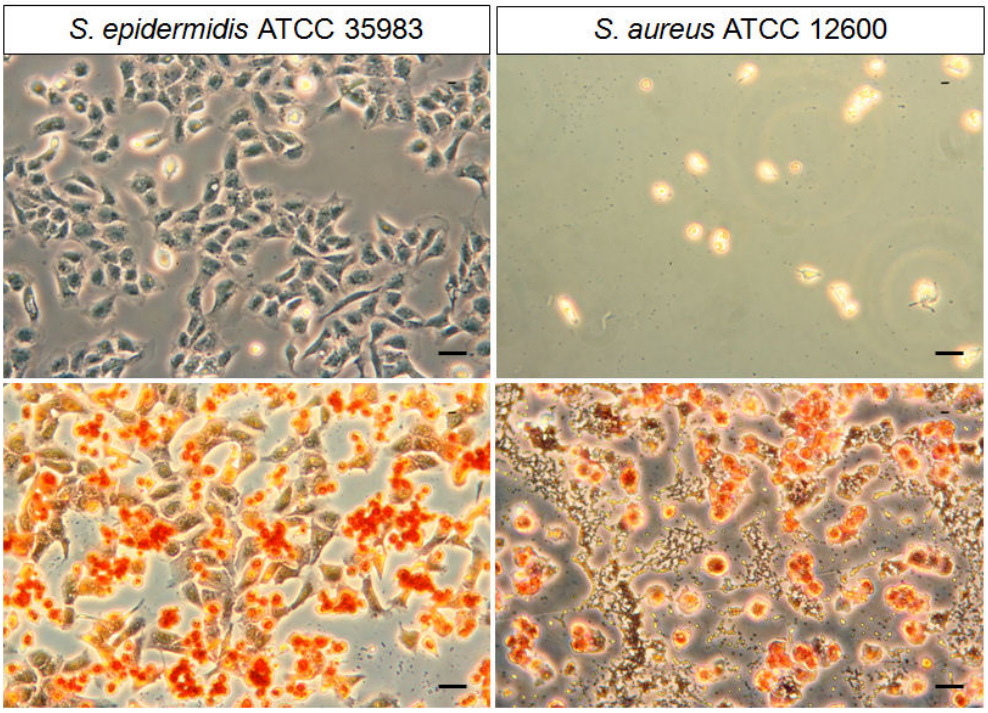

Figure 3. Phase-contrast images of adhered cells to PMMA after 24 h of simultaneous growth of U2OS and *S. epidermidis* ATCC 35983 or *S. aureus* ATCC 12600 in the absence (upper images) and presence (lower images) of macrophages. Macrophages are orange-stained. The bar denotes 50 µm.

Discussion

This paper presents the first experimental model to study the simultaneous interaction of macrophages-bacteria-osteoblasts on a biomaterial surface in a single experiment. In our *in vitro* model, bacteria were allowed to adhere prior to adhesion of macrophages and U2OS cells, which mimics a peri-operative bacterial contamination of implant surfaces. The number of bacteria adhering on the PMMA surface prior to macrophages and U2OS cell adhesion was set to 10^3 cm^{-2}. In the past, it has been documented that during a surgical procedure of 1 h, the total number of bacteria carrying particles falling on a wound is about 270 cm^{-2}. The bacterial counts were generally higher during periods of high activity and when more people were present in the operation theatre.[14]

Recently, through the use of modern, better ventilated operation theatres (20 changes of air/h) and impermeable patient and personnel clothing, peri-operative bacterial contamination is likely to be reduced.[15] However, many surgical procedures in which implants are introduced in the body last longer than 1 h. Therefore, the level of bacterial

contamination chosen in our experiments is probably realistic of a worst case scenario. Despite these low numbers, peri-operatively introduced organisms, particularly when of low virulence, can survive on an implant surface for prolonged periods of time and later, during periods of host immune depression, they proliferate and establish an infection with clinical symptoms.[16]

The pathogenesis of BAI is complex and depends on factors such as bacterial virulence, physicochemical properties of the biomaterial and alterations in the host defense.[17] Previously, in a model for the competition between bacteria and tissue cells, all common biomaterial surfaces, including PMMA, allowed *S. epidermidis* ATCC 35983 biofilm formation with a negative impact on the coverage of the biomaterial surface by tissue cells.[13] Yet, PMMA showed better cell adhesion and spreading in the presence of adhering *S. epidermidis* ATCC 35983 than other commonly used biomaterials.[13] Our present study supports previous observations that U2OS cells are able to adhere, spread and grow in the presence of *S. epidermidis* ATCC 35983, and extend these observations to the absence and presence of macrophages. On the other hand, in the presence of adhering *S. aureus* ATCC 12600, death of all adhering U2OS cells and macrophages within 18 h was observed despite the suspected removal of the majority of the bacterial toxins by flow. These observations are in line with clinical findings that BAI due to *S. aureus* usually progresses much more aggressively than BAI caused by *S. epidermidis*. In *S. epidermidis* infections, biofilm formation is considered the only virulence factor and therefore infections are usually sub-acute or chronic. The low virulence of *S. epidermidis* strains compared to *S. aureus* is due to the lack of additional genes responsible for producing severely tissue damaging toxins.[3,18]

In general, immune cells migrate, engulf and kill invading microorganisms.[19-21] A previous study on the interaction between macrophages and colonizing *S. epidermidis,* showed that macrophage behavior is surface dependent.[22] Macrophage migration towards bacteria and phagocytosis was enhanced on cross-linked poly(ethylene)-glycol (PEG) based polymer coatings compared to the uncoated substrata due to the weak adhesion of macrophages and bacteria to the PEG coating.[22] In our study, macrophages migrate towards the bacteria on a PMMA surface and engulfed the bacteria. The phagocytosis of bacteria by macrophages differs depending on the virulence of the strain.

In the presence of low virulent *S. epidermidis*, bacterial biofilm growth was strictly reduced by the presence of macrophages up to 20 h compared to only 14 h in the case of high virulent *S. aureus* biofilm growth. These results are in line with previous studies showing that in both *in vivo* and *in vitro* the uptake rate of bacteria by macrophages was inversely proportional to the virulence of the bacteria.[23,24] Furthermore, macrophages disintegration and necrotic death has been observed *in vitro* and *in vivo* due to overloading with ingested bacteria.[25,26] In this study it was observed that after a period of time macrophages become exhausted and break open which leads to a burst release of bacteria. At least part of these bacteria appeared to be active in the flow chamber. These findings suggest that J774 macrophages in this model are not able to kill all phagocytised bacteria. Although the viability of the released bacteria was not assessed, several studies have demonstrated that immune cells lose their ability to kill bacteria.[25,21,27,28] Leid *et al*.[29] showed that leukocytes were able to migrate to *S. aureus* biofilms but failed to phagocyte the bacteria. Neutrophils adjacent to Teflon cages, implanted in peritoneal cavities, exhibited decreased bactericidal activity and reduced superoxide production due to the increased production of *S. epidermidis* extracellular slime.[30-33] Watanabe *et al.*[28] demonstrated that engulfed *S. aureus* suppressed the production of superoxide, resulting in the prolonged survival inside the macrophages. In a murine model it was shown that high numbers of *S. epidermidis* could persist within macrophages in peri-catheter tissue without showing any signs of inflammation.[17] Also *S. epidermidis* inside macrophages were not only viable but were able to proliferate. *In vivo*, the local host defense was compromised because of the presence of biomaterials, resulting in deficient intracellular killing of pathogens by macrophages.[17]

The influence of macrophages on the competition between bacteria and mammalian cells is novel. This study demonstrates that despite the presence of macrophages, mammalian cells lost the race for the surface in the presence of high virulent *S. aureus*. *In vivo*, bacteria may well survive inside the macrophages for prolonged periods of time. These bacterial will favor the development of BAI, especially when certain physical conditions of the patients disturb the balance between bacteria and the host response.[17] This model validated for bacteria-macrophages-osteoblasts interactions in a flow chamber system resembles the *in vivo* environment more closely than single-cell type

cultures therewith providing an important bridge between *in vitro* and *in vivo* studies. Even though, this study was qualitatively analyzed, we believe that this methodology supported with quantitative data, could be a suitable tool for evaluation of biomaterials based on infection models.

References

1. Davis N, Curry A, Gambhir AK, Panigrahi H, Walker CR, Wilkins EG, Worsley MA, Kay PR. Intraoperative bacterial contamination in operations for joint replacement. *J Bone Joint Surg Br* 1999;81:886-889.
2. Hughes SP, Anderson FM. Infection in the operating room. *J Bone Joint Surg Br* 1999; 81:754-755.
3. Khalil H, Williams RJ, Stenbeck G, Henderson B, Meghji S, Nair SP. Invasion of bone cells by *Staphylococcus epidermidis*. *Microb Infect* 2007; 9:460-465.
4. Broekhuizen CA, De Boer L, Schipper K, Jones CD, Quadir S, Feldman RG, Dankert J, Vandenbroucke-Grauls CMJE, Weening JJ, Zaat SAJ. Peri-implant tissue is an important niche for *Staphylococcus epidermidis* in experimental biomaterial associated infection in mice. *Infect Immun* 2007; 75:1129–1136.
5. Vuong C, Otto M. *Staphylococcus epidermidis* infections. *Microb Infect* 2002; 4:481-489.
6. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* 1987; 237:1588-1595.
7. Anderson JM. Inflammation, wound healing, and the foreign-body response. pp 296-304. In: B.D. Ratner, A.S. Hoffman, F.J. Schoen and J.E. Lemons (ed.), Biomaterials Science. An Introduction to Materials in Medicine. 2004; Elsevier, San Diego, CA.
8. Xia Z, Triffitt JT. A review on macrophage responses to biomaterials. *Biomed Mater* 2006; 1:R1-9.
9. Berton G, Lowell CA. Integrin signaling in neutrophils and macrophages. *Cell Signal* 1999; 11:621-635.
10. Maddikeri RR, Tosatti S, Schuler M, Chessari S, Textor M, Richards RG, Harris LG. Reduced medical infection related bacterial strains adhesion on bioactive RGD modified titanium surfaces: A first step toward cell selective surfaces. *J Biomed Mater Res* 2008; 84A:425-435.
11. Shi ZL, Neoh KG, Kang ET, Poh C, Wang W. Bacterial adhesion and osteoblast function on titanium with surface-grafted chitosan and immobilized RGD peptide. *J Biomed Mater Res* 2008; 86A:865-872.

12. Subbiahdoss G, Kuijer R, Grijpma DW, Van der Mei HC, Busscher HJ. Microbial biofilm growth vs. tissue integration: "The race for the surface" experimentally studied. *Acta Biomater* 2009; 5:1399-1404.

13. Subbiahdoss G, Grijpma DW, Van der Mei HC, Busscher HJ, Kuijer R. Microbial biofilm growth vs. tissue integration on biomaterials with different wettabilities and a polymer – brush coating. *J Biomed Mater Res A* 2010;DOI:10.1002/jbm.a.32731.

14. Fitzgerald RH. Microbiologic environment of the conventional operating-room. *Arch Surg* 1979; 114:772-775.

15. Verkkala K, Eklund A, Ojajarvi J, Tiittanen L, Hoborn J, Makela P. The conventionally ventilated operating theatre and air contamination control during cardiac surgery - bacteriological and particulate matter control garment options for low level contamination. *Eur J Cardiothorac Sur*g 1998; 14:206-210.

16. Dougherty SH. Pathobiology of infection in prosthetic devices. *Rev Infect Dis* 1988; 10:1102-1117.

17. Boelens JJ, Dankert J, Murk JL, Weening JJ, Van der Poll T, Dingemans KP, Koole L, Laman JD, Zaat SAJ. Biomaterial-associated persistence of *Staphylococcus epidermidis* in pericatheter macrophages. J Infect Dis 2000; 181:1337-1349.

18. Massey RC, Horsburgh MJ, Lina G, Hook M, Recker M. Opinion - The evolution and maintenance of virulence in *Staphylococcus aureus*: a role for host-to-host transmission? *Nat Rev Microbiol* 2006; 4:953-958.

19. Babior BM. Oxidants from phagocytes: agents of defense and destruction. Blood 1984; 64:959-966.

20. Guenther F, Wabnitz GH, Stroh P, Prior B, Obst U, Samstag Y, Wagner C, Haensch GM. Host defence against *Staphylococcus aureus* biofilms infection: Phagocytosis of biofilms by polymorphonuclear neutrophils (PMN). *Mol Immun* 2009; 46:1805-1813.

21. Kaplan SS, Heine RP, Simmons RL. Defensins impair phagocytic killing by neutrophils in biomaterial-related infection. *Infect Immun* 1999; 67:1640-1645.

22. Saldarriaga Fernández IC, Da Silva Domingues JF, Van Kooten TG, Metzger S, Grainger DW, Busscher HJ, Van der Mei HC. Macrophage response to staphylococcal biofilm on cross-linked poly(ethylene) glycol polymer coatings *in vitro*. *Eur Cells Mater* 2010; Submitted.

23. Valtonen MV, Plosila M, Valtonen VV, Maekelae PH. Effect of the quality of lipopolysaccharide on mouse virulence of Salmonella enteritidis. *Infect Immun* 1975; 12:828-832.

24. Valtonen M Role of phagocytosis in mouse virulence of Salmonella enteritidis recombinants with O-antigen 6,7 or 4,12. *Infect Immun* 1977; 18:574-582.

25. Bonventre PF, Imhoff JG. Uptake of 3H-dihydrostreptomycin by macrophages in culture. *Infect Immun* 1970; 2:89-95.

26. Luhrmann A, Mauder N, Sydor T, Fernandez-Mora E, Schulze-Luehrmann J, Takai A, Haas S. Necrotic death of Rhodococcus equi-infected macrophages is regulated by virulence-associated plasmids. *Infect Immun* 2004; 72:853-862.

27. Tofte RW, Petersoson PK, Kim Y, Quie PG. Influence of serum concentration on opsonization by the classical and alternative complement pathways. *Infect Immun* 1980; 27:693-696.

28. Watanabe I, Ichiki M, Shiratsuchi A, Nakanishi Y. TLR2-Mediated survival of *Staphylococcus aureus* in macrophages: A novel bacterial strategy against host innate immunity. *J Immunol* 2007; 178:4917-4925.

29. Leid JG, Shirtliff HG, Costerton JW, Stoodley P. Human leukocytes adhere to, penetrate, and to respond to *Staphylococcus aureus* biofilms. *Infect Immun* 2002; 70:6339-6345.

30. Johnson GM, Lee DA, Regelmann WE. Interference with granulocyte function by *Staphylococcus epidermidis* slime. *Infect Immun* 1986; 54:13-20.

31. Myrvit QN, Wagner W, Barth E, Wood P, Gristina AG. Effects of extracellular slime produced by *Staphylococcus epidermidis* on oxidative responses of rabbit alveolar macrophages. *J Invest Surg* 1989; 2:381-389.

32. Shanbhag A, Yang J, Lilien J, Black J. Decreased neutrophil respiratory burst on exposure to cobaltchrome alloy and polystyrene *in vitro*. *J. Biomed Mater Res* 1992; 26:185-195.

33. Zimmerli W, Waldvogel F. Pathogenesis of foreign body infection. *J Clin Invest* 1984; 73:1191-1200.

Chapter 8

General Discussion

Biomaterial-associated infections (BAI) represent a considerable clinical caseload due to the high device and patient numbers and increasing complications (i.e. antibiotic resistance, persistence of infection in surrounding tissues) across all device categories. Associated high health care costs, burdens for infection mitigation, patient discomfort and not infrequently, death, present the motivation to design new solutions to this problem.

Bacterial adhesion to the surface of biomaterials and subsequent biofilm formation are crucial steps in the development of BAI, and therefore strategies to reduce the occurrence of these infections often aim to inhibit these processes. Examples of these strategies are poly(ethylene) glycol (PEG) coatings, which have been considered the most promising coatings due to their high capacity to inhibit adsorption of proteins and other biomolecules. Also, research on PEG-based coatings has clearly shown that bacterial adhesion is reduced by several orders of magnitude, but the fate of the few microorganisms that manage to adhere to PEG-based coatings is often ignored. It is therefore unknown whether these few adhering bacteria are capable to proliferate and form a clinically relevant infection. In this thesis we have aimed to take an extended approach to study different factors involved in the development of BAI on crosslinked poly(ethylene) glycol-based polymer coatings (OptiChem®).

BAI pathogenesis is a complex process in which surrounding tissue cells and the host immune system contribute to the clinical outcome of a bacterially contaminated biomaterial implant.[1] We tried to resemble *in vivo* circumstances by assessing bacterial and tissue cell adhesion and proliferation, and the response of macrophages to staphylococci adhering on OptiChem® coatings. Staphylococci were selected for the majority of the assessments as they are most commonly found in BAI on polymeric biomaterial surfaces. Furthermore, staphylococci showed the highest affinity for these coatings among the five isolates tested in chapter 3.

The adhesion of several clinical isolates to inert OptiChem® was reduced by more than 80%. This is several orders of magnitude larger than found on hydrophobic or positively charged coatings. *In vitro*, few adhering staphylococci managed to grow into biofilms when exposed to human blood plasma or macrophage culture media. Moreover, they adhered weakly and at a slow growth kinetics compared to uncoated substrata.

Interestingly, OptiChem® coated silicone rubber discs implanted in an infected pocket with *S. aureus* in mice did not become colonized within 5 days. This suggests that *in vivo* this strain is not capable to form a biofilm and subsequent infection on OptiChem® coated surfaces, despite our *in vitro* findings showing biofilm formation. We hypothesize that this is because OptiChem® was a good surface for the immune system, enabling more efficient clearing of the invading bacteria. Our *in vitro* study on the response of macrophages to colonizing bacteria on PEG-based coatings is a novelty. Indeed this study showed that macrophages on OptiChem® have a positive effect on the clearance of adhering staphylococci by having a high mobility and phagocyting bacteria efficiently. Thus this may explain why OptiChem®-coated silicone rubber discs showed to be efficient in limiting initial growth and in preventing recurrence of infection after revision surgery despite the fact that surrounding tissues were compromised.

Applications

OptiChem®, now commercially available as Slide H (Nexterion®) is a polymeric film that can be applied in a single step solvent-casting process with conventional techniques (spraying, dip-coating and spin coating) to a variety of substrata including glass, metal oxides and polymers.[2] This represents an advantage over other grafted or PEG-based coatings created by self-assembly, which are generally compatible with only specific, well-defined substrates and require prior surface modification, e.g. changing the surface charge or making the surface hydrophobic. Furthermore, OptiChem® coatings are chemically and physically thicker and more robust than common, grafted PEG coatings.[2] Coatings with such features may constitute an advantage in the context of medical implants because they allow more rigorous surgical handling and mechanical stresses, especially in the case of orthopedic devices, dental implants and surgical instrumentation.

In its activated form, OptiChem® coatings have an amine-reactive (NHS ester) terminal chemical functionality to allow specific immobilization of biomolecules *in situ*. The NHS esters can bind covalently with proteins and peptides that are recognized by tissue cells.

Therefore, when optimal tissue integration is required, for example in some orthopedic implants, cell binding peptides such as arginine-glycine-aspartic acid (RGD) can be immobilized on NHS-reactive OptiChem® coatings. RGD is an amino acid sequence recognized by many mammalian tissue cells via integrin receptors[2] and therefore it is anticipated that functionalization with these peptides may have a positive effect for many tissue cell types. For example, it has been demonstrated that immobilization of RGD on NHS-reactive OptiChem® coatings promotes fibroblast adhesion and proliferation.[3] Furthermore, bifunctional coatings containing RGD have been shown to have a high potential to reduce BAI as they supported osteoblast adhesion and proliferation while they strongly reduced bacterial adhesion.[4]

A weak point of PEG coatings in general is their instability, which limits their clinical application. X-ray photoelectron spectroscopy revealed that OptiChem® coatings remained stable and effective for at least 168 h after exposure to different biological fluids and different shear forces. The inert OptiChem® coatings may therefore be suitable for applications such as urinary catheters, voice prostheses, contact lense cases and dental implants.

Future research

Treatment of BAI involves antimicrobial therapy; therefore, the effect of incorporating antibiotics in the assessments is of significant importance. Previous research showed that gentamicin prevents staphylococcal biofilm growth on a PEO brush coated silicone rubber.[5] The effect of antibiotics on biofilms growing on OptiChem® is worth to be investigated. Furthermore, we showed that the proliferation of osteoblasts on NHS-reactive OptiChem® was suppressed in the presence of a staphylococcal biofilm. Whether tissue cells can proliferate in the presence of a bacterial biofilm treated with antibiotics would also be interesting to study. Also, it would be of interest to study biofilm formation and proliferation of osteoblasts on NHS-reactive OptiChem® equipped with RGD-sequences.

Bacteria and cells adhered to OptiChem® coatings were easily sheared-off by applying shear or through the passage of an air-bubble through the flow chamber. However, the exact force by which bacteria and cells adhere to these coatings is still unknown. Atomic force microscopy is a valuable tool to measure adhesion forces. Therefore this technique could be used to determine the adhesion strength of bacteria and cells under different circumstances, for example, different pH's and ionic strengths. Knowing the adhesion forces can contribute to the development of cleaning protocols for these coatings.

In this thesis, we included macrophages in the assessments and introduced a methodology to study the simultaneous interaction between bacteria, macrophages and osteoblasts. However, a limitation of this study was the killing ability of macrophages. There is evidence that the host immune system is impaired in the presence of a biomaterial, for example, by diminishing the phagocyting and killing ability. We showed that macrophages could phagocyte bacteria adhered to OptiChem® or PMMA; however, bacterial survival inside the macrophages was not assessed. This remains a question for future studies.

Conclusions

In summary, OptiChem® as a surface coating reduces bacterial adhesion in terms of numbers and adhesion strength. These weak interactions allow bacteria and biofilms to be removed easily from the coated surface by the immune system or applying high shear forces. In addition, this thesis shows the importance of looking beyond the prevention of bacterial adhesion and the use of appropriate *in vitro* models in order to predict the final outcome of modified biomaterials with surface coatings.

References

1. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* 1987; 237:1588-1595

2. Harbers GM, Emoto K, Greef C, Metzger SW, Woodward HN, Mascali JJ , Grainger DW, Lochhead MJ. Functionalized poly(ethylene glycol)-based bioassay surface chemistry that facilitates bio-immobilization and inhibits nonspecific protein, bacterial and mammalian cell adhesion. *Chem Mater* 2007;19:4405-4414

3. LeBaron RG, Athanasiou KA. Extracellular matrix cell adhesion peptides: functional applications in orthopedic materials. *Tissue Eng* 2000;6:85-103

4. Subbiahdoss G, Pidhatika B, Coullerez G, Charnley M, Kuijer R, Van der Mei HC, Textor M, Busscher HJ. Bacterial biofilm formation versus mammalian cell growth on titanium-based mono- and bi-functional coating. *Eur Cell Mater* 2010;13:205-213

5. Nejadnik MR. Polymer brush-coatings to prevent biomaterials associated infection: initial bacterial adhesion and biofilm formation. 2009 Dissertation, Rijksuniversiteit Groningen

Summary

Biomaterial associated infections (BAI) are a common complication associated with the use of indwelling devices. BAI threatens the device longevity and functionality and represents a serious health threat associated to high health care costs and increased morbidity and mortality. Therefore, novel biomaterials and coatings to make surfaces less prone to BAI are essential to develop.

In **Chapter 1**, an introduction to BAI and polymer brushes as a strategy to reduce the risk of BAI is outlined. Furthermore, this chapter presents the potential of a multicomponent crosslinked poly (ethylene) glycol-based coating (OptiChem®) for biomaterials. The general aim of this thesis was to investigate the extent up to which OptiChem® can contribute to the prevention of BAI.

The treatment of BAI is a challenge for clinicians and generally involves the removal of the compromised device followed by antimicrobial therapies to clear infection. The challenges posed by a BAI are highlighted in the study presented in Chapter 2. In **Chapter 2**, the fate of secondary implants is determined after antibiotic treatment of a BAI and surgical removal of the infected primary implant. Infected silicone rubber discs were implanted in a subcutaneous pocket in mice and BAI was routinely treated with antibiotics. After 4 days, the discs were removed and new sterile secondary silicone rubber discs were implanted. Implants as well as tissue samples were cultured for bacterial presence. All secondary silicone rubber discs and 80% of all tissue samples in the control group were colonized by bacteria. In the antibiotic treated group, 17% of all secondary discs and 33% of the tissue samples were culture-positive, despite antibiotic treatment. It is concluded that infection may persist in surrounding tissue lowering the prospects of a secondary implant, as they are at a greater risk for a BAI.

Bacterial adhesion to the surface of biomaterials is the first step in the pathogenesis of BAI and depends on many factors, which include the physicochemical surface characteristics from the interacting biomaterial and microorganisms, and the environment in which they interact (e.g. pH, temperature, medium, shear forces). In **Chapter 3**, five clinically isolated bacterial strains involved in BAI were used to evaluate the effect of inert OptiChem® coatings. Adhesion to the coatings was performed under laminar flow and at different shear rates. Bacterial adhesion to inert OptiChem® was reduced by more than 80% compared to glass. Inert OptiChem® was also exposed to

saliva, urine, blood plasma and PBS and its effectiveness to reduce the adhesion of *S. epidermidis* 3399 persisted for at least 168 h exposure to PBS or urine but not in protein-rich environments such as saliva and blood plasma. X-ray photoelectron spectroscopy indicated that coating integrity was not affected by exposure to any of these fluids.

Bacterial adhesion to polymer brushes is significantly reduced in terms of numbers and binding strength. However, few bacteria still adhere to these surfaces. It is unknown whether this low density of bacteria will eventually grow into a biofilm, which is the topic of Chapter 4.

Chapter 4 shows the kinetics of staphylococcal biofilm formation on inert OptiChem® *in vitro* and *in vivo*. Biofilm formation was inhibited *in vitro*, but when the coatings were exposed to plasma proteins, biofilm formation was observed. However, the kinetics of microbial growth were strongly delayed compared to the control surface. *In vivo*, inert OptiChem® coated silicone rubber discs inserted into an infected murine subcutaneous pocket did not become colonized upon reimplantation despite the fact that bacteria were present in the surrounding tissues. We conclude that OptiChem® coatings considerably slow down bacterial biofilm formation both *in vitro* and *in vivo*.

Another important factor for the success of implanted biomaterials is a long-term integration between the biomaterial surface and the surrounding tissue cells. When the biomaterial surface provides a good interface for host cells, cells can adhere and proliferate diminishing the chance of BAI. For this purpose, PEG coatings can be modified with molecules to favor tissue cell adhesion. In **Chapter 5,** growth and proliferation of U2OS osteosarcoma cells on glass, inert and amine-reactive OptiChem® coatings in the presence or absence of staphylococci was assessed simultaneously. Assessments were performed in a parallel plate flow chamber under laminar flow conditions at a low shear rate. Osteoblasts adhered and proliferated well on glass and on the reactive PEG-based coating during 48 h, but not on the inert coating. However, the effect of the NHS-activation in the reactive coatings was lost in the presence of adhering staphylococci. These findings demonstrate the importance of using bacterial and cellular co-cultures when assessing functionalized biomaterial coatings.

The presence of a biomaterial can affect the immune system's response to bacterial infection, and the biomaterial surface chemistry can stimulate or reduce macrophage adhesion, migration, and phagocytic activity. Therefore, macrophage-biomaterial-bacterial interactions are crucial in the pathogenesis of BAI. In **Chapter 6**, we introduce a novel methodology that enables direct, quantitative and detailed qualitative *in situ* observation of macrophage morphology, migration and phagocytosis of bacteria. This methodology was used to study the interaction between macrophages and *S. epidermidis* adhered to inert and NHS-reactive OptiChem®. Macrophage migration and bacterial clearance per macrophage were enhanced on OptiChem® coatings corresponding to removal of 60% of the adhered bacteria while on glass only 20% reduction was achieved. These findings suggest that bacterial clearance from OptiChem® coatings by macrophages is more effective than from glass, possibly as a result of a weak adhesion of bacteria on OptiChem®. Moreover, also macrophages are less immobilized on OptiChem® and therewith have the ability to clear a larger area from adhering bacteria than on glass, where they appear immobilized.

Chapter 7 presents an *in vitro* model to study the interaction between bacteria, macrophages and osteoblasts simultaneously on a biomaterial surface. This is an important contribution because the model provides a bridge between *in vitro* and *in vivo* experiments for the evaluation of BAI. In this model, we first adhere *S. aureus* or *S. epidermidis* to PMMA. Subsequently, U2OS and macrophages are seeded, and the adhesion and proliferation of bacteria and tissue cells in the presence of macrophages under low shear (0.14 s^{-1}) is evaluated simultaneously for 24 h in a single experiment. The presence of macrophages delayed biofilm growth. However, *S. aureus* biofilms induced death of osteoblasts whereas osteoblast adhesion and spreading was almost unaffected by *S. epidermidis* biofilms, irrespective of macrophage presence in the system. Important findings of this thesis are discussed in **Chapter 8**. Furthermore, we discuss the advantages of OptiChem® above other PEG-based coatings and indicate some applications for which OptiChem® may perform best as a biomaterial coating. Finally, suggestions for future research are given.

Samenvatting

Biomateriaal geassocieerde infecties (BAI) zijn een veel voorkomende complicatie bij het inbrengen en gebruik van medische implantaten in patiënten. BAI vermindert de levensduur en functionaliteit van de ingebrachte implantaten en vormen een serieuze bedreiging voor de gezondheid van de patiënt. BAI veroorzaakt daardoor hoge kosten voor de gezondheidszorg en een verhoogde morbiditeit en mortaliteit. Het is daarom essentieel dat nieuwe biomaterialen en coatings worden ontwikkeld die minder gevoelig zijn voor BAI.

In **hoofdstuk 1** wordt een introductie gegeven met betrekking tot BAI en het gebruik van polymeer borstels als een strategie om het risico op BAI te verminderen. Bovendien wordt in dit hoofdstuk de mogelijkheid van het gebruik van een op polymeer borstels gebaseerde coating (multicomponent crosslinked poly (ethylene) glycol-based coating, OptiChem®) om BAI te voorkomen, besproken. Het doel van dit proefschrift was te onderzoeken in welke mate OptiChem® kan bijdragen aan het verminderen van BAI.

De behandeling van BAI is een uitdaging voor de clinicus en bestaat meestal uit het verwijderen van een geïnfecteerd implantaat gevolgd door een antimicrobiële therapie om de infectie verder te bestrijden. De uitdagingen die gepaard gaan met de behandeling van BAI worden gepresenteerd in hoofdstuk 2. In **hoofdstuk 2** wordt de gevolgen van het inbrengen van een tweede implantaat bestudeerd nadat een BAI is behandeld met antibiotica en het eerste, geïnfecteerde implantaat is verwijderd. Siliconen rubberen schijfjes met een biofilm werden geïmplanteerd in een subcutane pocket in muizen. In een groep muizen, werd de BAI routinematig behandeld met antibiotica, en in de tweede groep met fysiologisch zout (controle groep). Na vier dagen werden de schijfjes verwijderd en werden nieuwe steriele siliconen rubberen schijfjes geïmplanteerd. De verwijderde siliconen rubberen schijfjes werden tezamen met weefselmonsters gekweekt om de aanwezigheid van bacteriën vast te stellen. Alle steriel ingebrachte siliconen rubberen schijfjes en 80% van alle weefselmonsters in de controle groep waren gekoloniseerd met bacteriën. In de groep behandeld met antibiotica was 17% van de kweken van de steriel ingebrachte schijfjes en 33% van de weefselmonsters positief, ondanks de antibiotica behandeling. De conclusie is dat in het geval van een BAI, de infectie in het omliggende weefsel kan binnendringen, waardoor de kansen van een

nieuw ingebracht implantaat worden verminderd, aangezien het risico op nieuwe BAI hierdoor is vergroot.

Bacteriële adhesie aan het oppervlak van biomaterialen is de eerste stap in de pathogenese van BAI en is afhankelijk van vele factoren, waaronder de fysisch-chemische oppervlakte karakteristieken van het biomateriaal en de micro-organismen én verschillende omgevingsfactoren, zoals pH, temperatuur, medium en aanwezigheid van afschuifkrachten. In **hoofdstuk 3** werden vijf klinisch geïsoleerde bacterie stammen betrokken bij BAI, gebruikt om het effect van een inerte OptiChem® coating te bestuderen. Bacteriële hechting aan de coating vond plaats onder een laminaire flow en bij verschillende afschuifkrachten in een parallelle plaat stroomkamer. Bacteriële hechting aan inert OptiChem® was meer dan 80% lager dan aan glas (controle). Inert OptiChem® werd ook blootgesteld aan speeksel, urine, plasma en PBS om de stabiliteit van de coating te bestuderen. De effectiviteit van OptiChem® in het verminderen van de hechting van *S. epidermidis* 3399 bleef bestaan na 168 uur blootstelling aan PBS of urine, maar de effectiviteit ging grotendeels verloren na blootstelling aan een eiwitrijk milieu zoals speeksel en plasma. Een elementen analyse van het oppervlak liet zien dat de integriteit van de coating niet werd aangetast door blootstelling aan een van deze vloeistoffen.

Bacteriële hechting aan polymeer borstels was significant verminderd t.o.v. de controle, zowel wat betreft het aantal bacteriën als wat betreft de bindingskracht van de bacterie met het oppervlak. Echter, enige hechting van bacteriën aan deze oppervlakken vindt nog steeds plaats. Het is onbekend of de bacteriën in deze lage dichtheid uiteindelijk zullen uitgroeien tot een biofilm. Dit is het onderwerp van hoofdstuk 4.

In **hoofdstuk 4** wordt de kinetiek van het ontstaan van een stafylokokken biofilm op inert OptiChem® bestudeerd zowel *in vitro* als *in vivo*. Het ontstaan van een biofilm op inert OptiChem® *in vitro* werd voorkomen, maar wanneer de coating werd blootgesteld aan plasma eiwitten kon vervolgens wel vorming van een biofilm worden waargenomen. Echter de kinetiek van de microbiële groei was fors vertraagd in vergelijking met de microbiële groei op glas (controle). *In vivo* raakten siliconen rubberen schijfjes gecoat met inert OptiChem®, die in een geïnfecteerde subcutane holte in een muis werden geïmplanteerd, niet gekoloniseerd, ondanks het feit dat in het omringende weefsel

bacteriën aanwezig waren. Wij concluderen dat OptiChem® coatings het ontstaan van een bacteriële biofilm aanzienlijk vertragen zowel *in vitro* als *in vivo.*

Een andere belangrijke factor die het succes van geïmplanteerde biomaterialen op lange termijn bepaalt is de integratie van het biomateriaal oppervlak in het omringende weefsel. Wanneer het oppervlak van een biomateriaal gunstige eigenschappen bezit voor interactie met de cellen van de gastheer, dan kan hechting en proliferatie van cellen plaatsvinden waardoor de kans op BAI afneemt. Om dit te bereiken kunnen polymeer borstel coatings worden gemodificeerd met moleculen die celhechting bevorderen. In **hoofdstuk 5** werd de groei en proliferatie van U2OS osteosarcoma cellen op glas, inerte en amino-reactieve OptiChem® coatings zowel in de aan- als afwezigheid van stafylokokken bestudeerd. Dit onderzoek gebeurde ook in een parallelle plaat stroomkamer bij laminaire stroming en een lage afschuifkracht. Gedurende de 48 uur dat er werd gemeten was er een goede hechting en proliferatie van de osteoblasten op glas en op de amino-reactieve coating, maar niet op de inerte coating. In de aanwezigheid van stafylokokken ging het effect van de amino-reactieve coating echter verloren. Deze bevindingen laten het belang zien van bacteriële en cellulaire co-culturen bij het onderzoeken van biomateriaal coatings met specifieke functionele groepen om weefselcel hechting te stimuleren.

De aanwezigheid van een biomateriaal kan de respons van het immuunsysteem op een bacteriële infectie beïnvloeden. De chemische eigenschappen van het biomateriaal oppervlak kan de hechting, migratie en de fagocytose van macrofagen stimuleren of remmen. De interactie tussen macrofagen, biomateriaal en bacteriën is daarom cruciaal in de pathogenese van BAI. In **hoofdstuk 6** introduceren we een nieuwe methode die het mogelijk maakt om *in situ* direct kwantitatieve en gedetailleerde kwalitatieve waarnemingen te doen van de macrofagen morfologie, migratie en fagocytose van bacteriën. Deze methode werd gebruikt om de interactie tussen macrofagen en *S. epidermidis* te bestuderen na hechting aan inert OptiChem® en amino-reactieve OptiChem®. De migratie van macrofagen en de bacteriële verwijdering per macrofaag namen toe op beide OptiChem® coatings, resulterend in een verwijdering van 70-80% van de gehechte bacteriën, terwijl op glas slechts 20% van de bacteriën werd verwijderd. Deze bevindingen suggereren dat de verwijdering van bacteriën op een

OptiChem® coating door macrofagen effectiever is dan op glas, mogelijk als gevolg van een zwakkere hechting van bacteriën aan OptiChem®. Bovendien zijn de macrofagen mobieler op OptiChem® en hebben ze daarmee de mogelijkheid om een groter gebied vrij te maken van bacteriën in vergelijking met glas, waarop de macrofagen meer geïmmobiliseerd lijken.

Hoofdstuk 7 presenteert een *in vitro* model om gelijktijdig de interactie tussen bacteriën, macrofagen en osteoblasten te bestuderen op het oppervlak van een biomateriaal. Dit is een belangrijk model omdat het een brug slaat tussen de huidige *in vitro* en *in vivo* experimenten ter evaluatie van BAI. In dit model lieten we eerst *S. aureus* of *S. epidermidis* hechten aan PMMA. Vervolgens werden U2OS cellen en macrofagen toegevoegd en werd de hechting en proliferatie van de bacteriën en U2OS cellen bij lage afschuifkracht (0.14 s^{-1}) in de aanwezigheid van de macrofagen bestudeerd gedurende 24 uur in één enkel experiment. De aanwezigheid van macrofagen remde het ontstaan van een biofilm. Een biofilm van *S. aureus* induceerde celdood van de osteoblasten, terwijl de hechting en spreiding van osteoblasten nauwelijks werd beïnvloed door een *S. epidermidis* biofilm. Dit effect was onafhankelijk van de aanwezigheid van macrofagen in het systeem.

De belangrijkste bevindingen van dit proefschrift worden besproken in **hoofdstuk 8**. Bovendien worden in dit hoofdstuk de voordelen van OptiChem® ten opzichte van andere op PEG gebaseerde coatings besproken en wordt ingegaan op enkele mogelijke applicaties waarvoor OptiChem® het meest geschikt is als coating van biomaterialen. Tot slot worden suggesties gegeven voor toekomstig onderzoek.

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